IMMUNOLOGICAL RELATIONSHIPS OF CELL CONSTITUENTS OF ENCAPSULATUS PNEUMONIÆ (FRIEDLÄNDER'S BACILLUS).

By LOUIS A. JULIANELLE, Ph.D.

(From the Hospital of The Rockefeller Institute for Medical Research.)

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A previous publication (1) on the biological classification of Friedländer's bacillus reported the existence of at least three specific types and a heterogeneous group among these bacilli. The groupings, designated as Types A, B, and C, and Group X, are sharply defined and highly specific by a number of immunological reactions. In a later communication (2) it was stated that encapsulated strains are usually virulent, produce soluble specific substance, and as antigen, induce the formation of type-specific antibodies which operate effectively both in test-tube reactions and in animal protection tests. Capsule-free strains, on the other hand, are avirulent, do not produce soluble specific substance, and as antigen stimulate only the undifferentiated species antibody. Type-specific antisera react irregularly with capsule-free strains; and the species-specific antisera, while reacting with capsule-free organisms regardless of type derivations, do not react at all with encapsulated cells.

The accumulated evidence on the serological reactions of Pneumococcus (3) and Friedländer's bacillus (1, 2, 4, 5) discloses that both species are composed of specific types which are referable to the elaboration of soluble specific substance by the organisms. Under certain conditions, the cells degrade into capsule-free bacteria which, among other changes, show lack of virulence and capsule formation, loss of elaboration of specific carbohydrate, and loss of type-specific antigenicity—all of which properties are the opposite of those which characterize their encapsulated antecedents. In virtue of the striking parallelism in the immunological behavior of Pneumococcus and Friedländer's bacillus it seemed of interest to project into the latter group the principles which govern the immunological relationships of the cell constituents of Pneumococcus.

The comparative immunological studies of the soluble specific substance and the nucleoprotein of Pneumococcus (3) reveal that the soluble specific substance, a nitrogen-free carbohydrate, reacts specifically with antipneumococcus serum of the homologous type. In the dissociated, dissolved, state, it does not serve as antigen; but in the form in which it exists in the cell it functions antigenically to produce type-specific antibodies. The nucleoprotein, on the other hand, is protein in nature and induces in the animal an antiserum which contains only the antiprotein or common, species antibody.

The present study is concerned with the immunological relationships of cell constituents of Friedländer's bacillus and the occurrence of these constituents in culture and body fluids of infected animals.

Methods.

The Soluble Specific Substance.—Methods for the fractionation of soluble specific substance of Friedländer's bacillus have been described in papers from this laboratory (5). It was shown at that time that carbohydrate derived from Strain E (Type B) is dextrorotatory, shows an acid equivalent varying from 670 to 716, is nitrogen-free, and on hydrolysis yields about 75 per cent reducing sugars. It reacts only in type-specific sera to a dilution of 1 to 4 million. Purified, nitrogen-free polysaccharides of Types A and B prepared in this laboratory were utilized in the present study through the courtesy of Drs. Heidelberger and Goebel.

The Nucleoprotein.—Several methods were employed for the separation of the protein and none of the methods were entirely satisfactory. The yield was usually small and the solutions underwent denaturation on keeping. The method finally adopted, however, made use of non-encapsulated cells (since no difference could be shown between the protein derived from encapsulated and capsule-free cells, respectively). The growth from the surface of the agar in Blake bottles was washed off in sterile distilled H_2O. To this suspension NaOH was added to an ultimate concentration of .005 N. The suspensions were frozen and thawed successively a dozen times or more and then diluted 3–4 times with distilled H_2O and centrifuged. The resultant supernatant was filtered, so that a sterile cell-free filtrate was obtained. Precipitation was then effected with a minimum amount of N acetic acid and the precipitate was whirled down. The supernatant was discarded and the precipitate was redissolved in a minimum amount of .01 N NaOH. Usually acid precipitation and solution with alkali were repeated and the final product was made up in .85 per cent NaCl. All protein solutions were standardized on the basis of nitrogen content.

Immunological Reactions.—The method of immunization, the reactions of agglutination and precipitation and protection test have been described in an earlier paper (1).

EXPERIMENTAL.

I. The Soluble Specific Substance.—(*a*) *Antigenic Properties.*—Repeated observations already published from this laboratory leave no doubt that the chemically purified polysaccharide of Pneumococcus is non-antigenic. Similar studies (6) of Zinsser, Mueller, and their associates also record the lack of antigenicity of "residue antigen" from a number of bacterial species. The "residue antigen" of these investigators is a substance which is extracted from bacteria and which bears a definite relation to the specific character of the bacterial cell.

In the present study observations on the antigenicity of the carbohydrate of Friedländer's bacillus are confined to the immunization of rabbits with the polysaccharide derived from Group X. In this instance a solution of bacterial cells was prepared from an encapsulated strain and it consequently contained dissociated soluble specific substance. Immunization with this product even in the presence of nucleoprotein yielded no specific antibodies, as will be pointed out later. Since the results from various sources indicate that bacterial polysaccharides are not antigenic, the lack of specific antibody response to a solution containing both protein and carbohydrate is evidence of similar conditions in the case of Friedländer's bacillus, also. At the same time it is clear that bacterial dissolution is accompanied by antigenic dissociation.

(*b*) *Serological Properties.*—It has been previously demonstrated (1, 2, 4, 5) that the polysaccharides derived from Friedländer's bacillus react specifically with immune sera of the homologous type. (*Cf.* in this connection Table IV.) In fact, sufficiently conclusive proof has been presented to show that just as has been shown with Pneumococcus, the soluble specific substance confers upon the cell its immunological type specificity.

II. The Nucleoprotein.—(*A*) *Antigenic Properties.*—It is realized that the acetic acid-precipitable material represents more than the nucleoprotein of the bacterial cell, and that it is a mixture of proteins rather than a single antigenic unit. Nevertheless, for the purposes of the present study, this fact offers no difficulty in either the performance or interpretation of the various reactions employed. Rab-

TABLE I.

Agglutination of "S" Strains of Friedländer's Bacillus by Anti-P Sera.

Antigen encapsulated strain	Anti-P sera derived from									Anti-S sera—Type			
	Type A			Type B			Group X			A	B	C	X
	*1:1	1:5	1:10	1:1	1:5	1:10	1:1	1:5	1:10	1:5	1:5	1:5	1:5
Type A	−	−	−	−	−	−	−	−	−	++++	−	−	−
" B	−	−	−	−	−	−	−	−	−	−	++++	−	−
" C	−	−	−	−	−	−	−	−	−	−	−	++++	−
Group X	−	−	−	−	−	−	−	−	−	−	−	−	++++

++++ indicates complete, disc agglutination.

* The figures represent dilution of serum.

TABLE II.

Agglutination of "R" Strains of Friedländer's Bacillus by Anti-P Sera.

Anti-P sera	Protein antigen from	Final dilution of serum							Normal serum 1:5
		1:5	1:10	1:20	1:40	1:80	1:160	1:320	
Type A	Type A	++++	++++	++++	++++	++++	++++	++++	−
	" B	++++	++++	++++	+++	+++	++	++	−
	" C	++++	++++	+++	+++	++	+	+	−
	Group X	++++	++++	++++	++++	++++	++++	++++	−
" B	Type A	++++	++++	++++	++++	++++	+++	++	−
	" B	++++	++++	++++	++++	++++	+++	++	−
	" C	++++	++++	++++	+++	+++	++	++	−
	Group X	++++	++++	++++	++++	++++	++++	++++	−
Group X	Type A	++++	++++	++++	+++	++	+	+	−
	" B	++++	++++	++++	++++	++++	+++	++	−
	" C	++++	++++	+++	+++	++	++	+	−
	Group X	++++	++++	++++	++++	++++	++	+	−

++++ indicates complete agglutination; +++, marked agglutination, ++, moderate agglutination; +, slight agglutination.

bits were immunized to the protein isolated from representative strains of Types A and B and Group X. The immune sera were tested for agglutinins, precipitins, and protective antibodies.

(*a*) *Agglutinins.*—Antiprotein sera do not contain type-specific

agglutinins for encapsulated strains of Friedländer's bacillus. Evidence for this statement is found in Table I where it is seen that antisera prepared by immunization with protein derived from serologically different strains do not react with the encapsulated cell of either homologous or heterologous type.

It will be recalled that capsule-free (R) strains of Friedländer's bacillus (2) stimulate the formation of the common, undifferentiated

TABLE III.

Agglutination by Anti-P Sera of Suspensions of Friedländer's Bacillus Decapsulated by Porges' Method.

Anti-P serum derived from	Antigen derived from	Dilution of serum				
		1:50	1:100	1:250	2:500	1:1000
Type A	Type A	+++	+++	++++	++++	+++
	" B	++++	++++	++++	++++	+++
	" C	++	+++	+++	++	+
	Group X	+++	+++	++++	++++	+++
	Gran.*	++	++	+	−	−
" B	Type A	++	+++	++++	++++	++++
	" B	++++	++++	++++	+++	++
	" C	++	+++	+++	++	+
	Group X	+++	++++	++++	++++	+++
	Gran.	++	++	+	−	−
Group X	Type A	+	++	++++	++++	++++
	" B	++++	++++	++++	+++	+
	" C	+	+	+++	++	++
	Group X	++	+++	++++	++++	+++
	Gran.	++	++	++	+	−

* This organism is a strain of granuloma bacillus—isolated from an infection of granuloma inguinale.

species antibody; but that they are unable to provoke type-specific antibodies. Accordingly, it seemed pertinent to determine the reaction of antiprotein sera on the non-encapsulated strains. The results of the reactions are given in Table II. It is seen that capsule-free cells derived from any of the serologically different types agglutinate equally well in all the antiprotein sera. The agglutination is

characteristic of the R cells (2) and occurs at a high dilution of serum. Antiprotein sera, in other words, behave in this respect similarly to anti-R sera. It was to be expected, then, that antiprotein sera would agglutinate, also, suspensions of Friedländer's bacilli after decapsulation by Porges' method. Earlier observations (2) pointed out the serological similarity of capsule-free strains obtained by cultural and chemical means. Table III reveals that suspensions of encapsulated cells treated as Porges recommends are agglutinated in antiprotein sera just as are the "R" strains.

TABLE IV.

Precipitation of the Soluble Specific Substance of Friedländer's Bacillus by Anti-P Sera.

Serum	Soluble specific substance of Friedländer's bacillus											
	Type A						Type B					
	2	20	50	100	250	500	2	20	50	100	250	500
Type A (P)	−	−	−	−	−	−	−	−	−	−	−	−
" B "	−	−	−	−	−	−	−	−	−	−	−	−
" C "	−	−	−	−	−	−	−	−	−	−	−	−
Group X "	−	−	−	−	−	−	−	−	−	−	−	−
Type A (S)	−	+	++	++	+	−	−	−	−	−	−	−
" B "	−	−	−	−	−	−	−	++	+++	++++	+++	++
Normal	−	−	−	−	−	−	−	−	−	−	−	−

+++ indicates compact disc precipitation with clear supernatant; +++, marked disc precipitate; ++, thin film-like scale; +, ground glass turbidity.

The figures represent dilution in thousands.

(*b*) *Precipitins.*—It has been shown that type-specific precipitins are induced only by the encapsulated cell. Added confirmation of this fact is derived from the observation that antiprotein sera do not react with the specific polysaccharides of Friedländer's bacillus. This is evident from the results presented in Table IV. It is definite that none of the antiprotein sera are able to cause precipitation of carbohydrate isolated from strains of Type A or B.

That antiprotein sera react with capsule-free strains of Friedländer's bacillus constitutes direct evidence of the presence of the species antibody. That the species antibody is in reality an antiprotein

TABLE V.

Precipitation by Anti-P Sera of the "Nucleoprotein" of Friedländer's Bacillus and Allied Organisms.

Antigen*	Anti-P serum from	Ultimate dilution of protein						
		200	1000	2000	4000	8000	16,000	32,000
Type A protein	Type A	+++	+++	++	++	+	±	−
	" B	+++	+++	++++	+++	+++	+++	++
	Group X	+++	+++	++	+	−	−	−
		500	2500	5000	10,000	20,000	40,000	80,000
Type B protein	Type A	++++	++++	+++	++	+	±	−
	" B	++	++	+++	+++	+++	+++	+
	Group X	+++	+++	++	+	±	−	−
		200	1000	2000	4000	8000	16,000	32,000
Type C protein	Type A	++	++	++	+	+	±	−
	" B	++	++++	++++	++++	+++	+++	+
	Group X	−	+	++	++	+	−	−
		500	2500	5000	10,000	20,000	40,000	80,000
Group X protein	Type A	++++	++++	+++	++	+	±	−
	" B	++	++	+++	+++	++	++	±
	Group X	++	++	+	+	±	−	−
		500	2500	5000	10,000	20,000	40,000	80,000
B. aerogenes protein	Type A	++	+	+	±	−	−	−
	" B	++	+	+	±	−	−	−
	Group X	++	+	±	−	−	−	−
		300	1500	3000	6000	12,000	24,000	48,000
B. coli protein	Type A	++	+	+	±	−	−	−
	" B	++	++	+	+	±	−	−
	Group X	++	++	+	+	−	−	−
		200	1000	2000	4000	8000	16,000	32,000
Granuloma bacillus protein	Type A	++	++	+	+	−	−	−
	" B	++	++	++	+	+	−	−
	Group X	+	+	±	−	−	−	−

++++ indicates heavy precipitation, supernatant clear; +++, marked precipitation with cloud; ++, marked cloud, no precipitation; +, cloud; ±, faint cloud.

* None of the proteins were precipitated by normal serum.

antibody is evidenced by the reaction of protein precipitation in antiprotein sera. The results of these reactions are presented in Table V. It is seen that sera prepared against protein isolated from three serologically different strains cause the precipitation of protein derived from any of the four different strains. It becomes obvious therefore that nucleoprotein induces the formation of species antibodies which cause agglutination of capsule-free cells and precipitation of protein and that the reactions exhibit none of the type relationships.

In addition, Table V reveals that antiprotein sera of Friedländer's bacillus reacts with protein derived from *B. aerogenes*, *B. coli*, and granuloma bacillus. Stated in another way the protein of Fried-

TABLE VI.

Protection Offered by Anti-P Sera against Infection with Friedländer's Bacillus (Type A).

Type A encapsulated culture	Antiserum derived from				Virulence controls
	Type A		Type B		
	Amount	Result	Amount	Result	
cc.	*cc.*		*cc.*		
.001	.2	D. 20	.2	D. 19	
.0001	.2	" 16	.2	" 15	
.00001	.2	" 39	.2	" 16	D. 24
.000001	.2	" 39	.2	" 43	" 39
.0000001					" 65

D. indicates death, the numerals representing the number of hours before death occurred.

länder's bacillus bears a definite serological relationship to proteins of closely allied species. This lends considerable assistance in the interpretation of the results of former investigators who have observed that anti-Friedländer sera caused agglutination of *B. rhinoscleromatis* (7–10), *B. aerogenes* (11), typhoid (12), and granuloma bacillus (13), etc. The explanation of such cross-agglutinations appears to depend upon the fact that immunization with non-encapsulated strains or prolonged immunization with encapsulated strains stimulates the formation of agglutinins which act not only on R cells

of Friedländer's bacillus, but on R cells of closely related species, as pointed out above.

III. Protection.—Immune sera prepared by immunization with encapsulated Friedländer's bacilli confer upon white mice specific protection against infection by strains of the homologous type (1).

TABLE VII.

Protection Offered by Anti-P Sera against Infection by Friedländer's Bacillus (Type B).

Type B encapsulated culture	Anti-P serum derived from				Virulence controls
	Type A		Type B		
	Amount	Result	Amount	Result	
cc.	*cc.*		*cc.*		
.001	.2	D. 16	.2	D. 15	
.0001	.2	" 16	.2	" 15	
.00001	.2	" 39	.2	" 20	D. 16
.000001	.2	" 39	.2	" 67	" 39
.0000001					" 39

D. indicates death, the numerals representing the number of hours before death occurred.

TABLE VIII.

Precipitation of the Nucleoprotein of Friedländer's Bacillus by Anti-S Sera.

Anti-S Friedländer sera	Friedländer protein derived from							
	Type A		Type B		Type C		Group X	
	*600	6000	1200	12,000	900	9000	500	5000
Type A	++	+	+	++	++	+	++	+
" B	+	−	+	−	±	−	+	−
" C	++	+	++	+	++	+	++	+
Group X	+	−	++	−	+	±	+	−

* These figures represent the dilution of protein.

Immunization with non-encapsulated cells, however, yields no protective antibodies (2). Protective substances, therefore, accompany type-specific antibodies. It was anticipated then that lacking type-specific agglutinins and precipitins, Friedländer antiprotein sera would afford no passive protection against active infection. That this is

actually the case is borne out by the data given in Tables VI and VII. The lack of any protective action by antiprotein sera against infection with virulent strains of homologous and heterologous types is striking.

(*b*) *Serological Properties.*—

1. Precipitation of Protein in Anti-S Sera.—Anti-Friedländer sera resulting from immunization with encapsulated strains are dominantly type-specific. Consequently anti-S sera contain negligible amounts

TABLE IX.

Precipitation by Anti-R Sera of the "Nucleoprotein" of Friedländer's Bacillus.

Antigen	Anti-R serum from	Dilution of protein						
		200	1000	2000	4000	8000	16,000	32,000
Type A protein	Type A	+++	+++	++	++	+	−	−
	" B	+++	++	++	+	−	−	−
	Group X	++	+	+	±	−	−	−
		500	2500	5000	10,000	20,000	40,000	80,000
Type B protein	Type A	+++	++	+	−	−	−	−
	" B	++	++	+	−	−	−	−
	Group X	++	+	+	−	−	−	−
		200	1000	2000	4000	8000	16,000	32,000
Type C protein	Type A	++	+	+	−	−	−	−
	" B	++	+	+	−	−	−	−
	Group X	++	+	+	±	−	−	−
		500	2500	5000	10,000	20,000	40,000	80,000
Group X protein	Type A	+++	++	+	−	−	−	−
	" B	++	++	+	−	−	−	−
	Group X	+++	+++	++	+	−	−	−

of antiprotein as has been pointed out previously (2) by the agglutination of R cells in anti-S sera. In virtue of the presence of antiprotein, such sera may cause precipitation of protein. This fact is illustrated in Table VIII. Type A anti-Friedländer serum definitely contains the species antibody, while the remaining type sera contain traces or none at all.

2. Precipitation of Protein in Anti-R Sera.—Capsule-free strains of Friedländer's bacillus are agglutinated in antiprotein sera (Table

II). Evidence of the reciprocal nature of this reaction was obtained in the precipitation of protein by anti-R sera. It is seen from the results presented in Table IX that anti-R sera cause the precipitation of protein from Friedländer's bacillus of the different types. The conclusion can be drawn, therefore, that the common species antibody reacts with the undifferentiated antigen of Friedländer's bacillus whether the antigen is in the form of non-encapsulated cells or in the form of dissolved protein.

TABLE X.

Precipitation of Friedländer Protein by Anti-P Sera after Adsorption of the Species Antibody by R Strains.

Antigen protein derived from	Anti-P serum (Type A) after adsorption by R strains derived from encapsulated strains of											
	Type A			Type B			Type C			Group X		
	*1:200	1:2000	1:8000	1:500	1:5000	1:10,000	1:200	1:2000	1:8000	1:500	1:5000	1:8000
Type A	—	—	—	—	—	—	—	—	—	—	—	—
" B	—	—	—	—	—	—	—	—	—	—	—	—
" C	—	—	—	—	—	—	—	—	—	—	—	—
Group X	—	—	—	—	—	—	—	—	—	—	—	—

* The figures represent the dilution of protein.

(*c*) *Precipitation of Protein in Antiprotein Sera.*—It has been shown above that protein derived from any of the serological types is precipitated in all antiprotein sera. (*Cf.* Table IV.)

Adsorption of Antibodies in Antiprotein Sera by Non-Encapsulated (R) Strains of Friedländer's Bacillus.

Because agglutination of R cells occurs in antiprotein sera, and precipitation of protein is obtained in anti-R sera, experiments were conducted to gain information concerning the identity of the antibody involved in both reactions. Each antiprotein serum was adsorbed with heat-killed suspensions of R cells derived from encapsulated strains of Types A, B, and C, and Group X. Adsorption was continued until all the agglutinins were removed for the adsorbing

strain. It was found that removal of agglutinins by one strain adsorbed the agglutinins for the remaining R strains also. Moreover, the loss of agglutinins by adsorption was accompanied by a loss of precipitins. A typical protocol is presented in Table X. It is clear that adsorption of a serum resulting from immunization with protein derived from a strain of Type A, for example, with any of the four "R" strains deprives the serum of precipitins for protein derived from any of the types. It appears therefore that the undifferentiated species antibody is the same antibody whether it occurs in anti-R or antiprotein sera.

TABLE XI.

Occurrence of Soluble Specific Substance in Culture Filtrate. Friedländer Bacilli.

Strain	Dilution of filtrates															
	After 4 hrs.				After 8 hrs.				After 12 hrs.				After 24 hrs.			
	1:1	1:5	1:20	1:50	1:1	1:5	1:20	1:50	1:1	1:5	1:20	1:50	1:1	1:5	1:20	1:50
Type A.....	+	−	−	−	+	+	−	−	+++	++	+	−	++++	++++	++	+
" B.....	−	−	−	−	±	−	−	−	+	+	−	−	++++	+++	+	−
" C.....	+	−	−	−	++	+	−	−	++	++	+	−	++++	++++	+	−
Group X.....	±	−	−	−	++	+	−	−	++	+	−	−	++++	++	+	−

Occurrence of Soluble Specific Substance in Culture Filtrates of Friedländer's Bacillus.

It has been shown by Dochez and Avery (14) that the soluble specific substance of Pneumococcus is demonstrable in culture filtrates, and that the progressive increase of the carbohydrate bears a striking relation to the growth curve of the culture. Their results show clearly that the soluble specific substance is a product of metabolic activity rather than a product of cell disintegration.

In similar fashion, the polysaccharide of Friedländer's bacillus is demonstrable in actively growing cultures. Cell-free filtrates obtained at different intervals during the growth are precipitated specifically by anti-Friedländer sera. The data submitted in Table XI show that in some instances specific carbohydrate is present in filtrates as early as 4 hours after growth has been initiated. The

amount of the carbohydrate increases rapidly so that after 24 hours' growth a definite reaction may be obtained in culture filtrate in dilution of 1:20, and in one case (Type A) even in a dilution of 1:50.

Occurrence of Protein in Culture Filtrates of Friedländer's Bacillus.

The type specificity of anti-Friedländer sera depends in part upon the integrity, and the absence of R cells in the culture used for immunization. It becomes of importance, therefore, to determine the rapidity of the disintegration of Friedländer's bacilli. Dissociation may be estimated by the presence of the common or protein antigen. Accordingly an analysis of the protein content of culture filtrates of Friedländer's bacillus was made by the usual protein pre-

TABLE XII.

Occurrence of Protein in Culture Filtrates of Friedländer Bacilli.

Strain	Dilution of filtrates											
	At 12 hrs.		At 24 hrs.		At 72 hrs.		5 days		9 days			
	1:1	1:10	1:1	1:10	1:1	1:10	1:1	1:10	1:1	1:5	1:10	1:20
Type A	−	−	−	−	−	−	−	−	+	±	−	−
" B	−	−	−	−	−	−	−	−	++	+±	+	−
" C	−	−	−	−	−	−	−	−	++	+	±	−
Group X	−	−	−	−	−	−	−	−	++	+	±	−

cipitation test. As brought out in Table XII, no protein was demonstrated in culture filtrates after 5 days' growth. On the 9th day, precipitation of protein was obtained in all the filtrates studied. In contrast to the carbohydrate which is elaborated during the period of active growth, the protein is demonstrable after this period and when cell disintegration takes place.

This fact becomes of great significance in immunization with suspensions of Friedländer's bacillus. Despite the fact that both disintegration and R cells are absent in the cultures used for immunization, anti-Friedländer sera may contain variable amounts of protein antibody. This is evidence that the body defenses not only engender type-specific antibodies, but also include a mechanism which causes a cleavage or disintegration of the specific antigen.

Occurrence of Soluble Specific Substance in Friedländer Infections.

The soluble specific substance of Pneumococcus has been demonstrated in the serum and urine of patients during pneumonia by Dochez and Avery (14). Blake (15) has shown that this is also a fact in pneumonia due to Friedländer's bacillus. This is the only reference of its kind concerning Friedländer infection which has come to our attention. In the present study experiments were performed to detect specific carbohydrates in rabbits infected by intraperitoneal injections of Friedländer's bacilli. The urine and blood of the infected rabbits were collected and tested for soluble specific substance

TABLE XIII.

Occurrence of Soluble Specific Substance in the Blood and Urine of Animals Infected with Friedländer's Bacilli.

Type of infection	Body fluid	Dilution of fluid		
		1:1	1:5	1:10
Type B	Serum	++	+	−
	Urine	+	−	−
" C	Serum	+++	++	+
	Urine	++	+	−

The precipitin reaction was obtained only in homologous immune serum.

by the usual precipitin technique. It is seen from Table XIII that the specific polysaccharide of Friedländer bacilli is present in both urine and serum of rabbits, and is demonstrable within 18 hours after infection.

DISCUSSION.

The soluble specific substance of Friedländer's bacillus endows the cell with type specificity, and is separable from the bacterial cell as a pure, nitrogen-free polysaccharide. When dissociated from the cell, it does not function as antigen, but in the form in which it exists in the cell it stimulates the formation of antibodies which cause type-specific agglutination of encapsulated cells, precipitate the carbohydrate derived from organisms of the homologous type, and afford

passive protection in white mice against infection by bacilli of the same type. As reacting substance, it is precipitated only in antisera resulting from immunization with encapsulated strains from which it is derived.

The nucleoprotein is separable from dissolved Friedländer's bacilli by precipitation with acetic acid in the cold. This constituent differs in nature and in serological behavior from the soluble specific substance. It is protein in nature and is a common, undifferentiated constituent of all types. It is antigenic and provokes in the animal the common protein or species antibody. The species antibody does not react with encapsulated bacilli of any type nor with the soluble specific substance of either homologous or heterologous types, and does not protect against infection with Friedländer's bacillus. Anti-protein sera, however, cause agglutination of capsule-free cells derived from any of the serological types by either cultural or chemical methods; and they react also with protein from all types. Moreover, the protein antibody is of a sufficiently general nature to react with protein from allied bacteria. In this fact resides the explanation for the confusing cross-agglutination reactions obtained with related organisms by former workers. That the protein antibody is of more or less common occurrence among other species of bacteria gains evidence from the results of numerous investigators. The studies from this laboratory show this with Pneumococcus, and the work of Lancefield (16), Hitchcock (17), and Tunnicliff (18) discloses a distinct serological relationship between various species of the Gram-positive cocci. The contributions of Dopter (19) and Eberson (20) depict similar relationships among the Gram-negative cocci, and Smith and TenBroeck (21) and Felix (22) offer comparable data for members of the typhoid-colon group.

Antisera prepared by immunization with protein or with a degraded non-encapsulated R culture contain antibodies which are identical in their immunological reactions.

The presence of soluble specific substance in filtrates of growing cultures suggests that it is a product of growth activity of the cell. The presence of protein in filtrates of old cultures only, indicates that it is a product of cell autolysis and disintegration.

CONCLUSIONS.

1. The soluble specific substance of Friedländer's bacillus is non-antigenic when dissociated from the cell. It is different for each type and it is highly reactive in the corresponding anti-S serum.

2. The nucleoprotein is antigenic, induces the species or protein antibody which reacts with capsule-free cells and protein derived from all types. Antiprotein sera do not react with either the encapsulated cell or the polysaccharide derived from it, and they offer no protection against infection.

3. Anti-R and antiprotein sera are identical in their behavior.

4. The carbohydrate of Friedländer's bacillus is demonstrable in filtrates of actively growing cultures and in the blood and urine of infected animals.

5. The protein is demonstrable in filtrates of only old, disintegrating cultures.

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AN EXPERIMENTAL ANALYSIS OF BACTERIAL ALLERGY.

By HANS ZINSSER, M.D., and TAKEO TAMIYA, M.D.

(*From the Department of Bacteriology and Immunology, Harvard University Medical School, Boston.*)

Plates 29 and 30.

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I.

In the study of hypersusceptibilities a differentiation has been gradually developed between the phenomena concerned in true anaphylaxis to the coagulable proteins, in which it is generally assumed that a true antigen-antibody union upon the cells of the body underlies the responsible mechanism, and the many other manifestations of hypersusceptibility in which no such specific antigen-antibody relationship has been demonstrated. In the case of the bacterial hypersusceptibilities it is quite generally accepted that true protein anaphylaxis plays a relatively unimportant rôle in the occurrences of spontaneous infection, but there are other manifestations, spoken of as bacterial allergy, which are recognized both by systemic and local reactions, and which are of considerable importance diagnostically and perhaps pathologically and prognostically. The mechanism of these conditions has not so far been clearly understood.

In our own studies we have occupied ourselves for a number of years chiefly with the tuberculin reaction, since this phenomenon represents the most extreme example of bacterial allergy and is easily amenable to experimental study. Since, however, we encountered considerable experimental difficulties in elucidating the mechanism of the tuberculin reaction by direct attack, we were forced to extend our studies to experiments with many different bacteria, choosing, in addition to tubercle bacilli and their products, such organisms as the staphylococcus, streptococcus, typhoid bacillus, *Bacillus abortus bovis* and pneumococcus, thus including organisms of many degrees of solubility

and of far greater potency for free antibody production than *Bacillus tuberculosis*.

The premises from which the work reported in the present paper takes its departure, some of them elucidated by our associates and ourselves, many of them either suggested or independently worked out by others, may be briefly stated as follows:

1. The tuberculin reaction is independent of general anaphylaxis to tuberculoprotein. (This was quite clear from our early paper (1), which confirmed by other methods the suggestions made long before by Baldwin (2) and by Krause (3), and confirmed also by Selter (4) and by Bessau (5).)

2. Tuberculin hypersensitiveness in its typical and extreme form cannot be induced by dissolved extracts of the tubercle bacillus provided that these are filtered through Berkefeld filters to remove formed particles, but it can be characteristically induced not only by infection with the living bacilli, but by treatment with dead organisms, even when these are boiled (6). These facts confirm the importance of typical tissue reactions spoken of as tubercles in the mechanism of tuberculin sensitization.

3. The mechanism of tuberculin reactions is independent of the presence in the animal body of those precipitating or agglutinating antibodies which act either upon the whole bacilli or on the precipitable carbohydrate "residue" antigen *in vitro*.

4. Conversely, the substance in tuberculin which is responsible for reaction in the sensitized animal is chemically separable from the precipitable residue carbohydrate fraction which reacts with antibodies *in vitro*, and is probably a nitrogenous substance, perhaps a form of protein (Mueller (7); Laidlaw and Dudley (8)).

There are many other minor facts which we have emphasized in preceding papers and which might be included in the premises from which we have worked, but these have been discussed by us in other places and will appear, where important, in other parts of this communication.

The difficulties of working with bacterial allergy have been manifold, chiefly because the bacterial cell is chemically complex, often possessing primary toxicity which produces reactions that are not easily distinguished from allergy in the sensitized.

Also, there have been difficulties in the choice of the proper animals for experimental purposes in that, here even more than in protein anaphylaxis, fundamental differences exist in the mechanism by which different species of animals respond to sensitization. The primary toxicity of many of the bacterial materials, and the necessity of often working with suspensions rather than with true solutions, render unreliable intravenous injection, with constitutional allergic responses, and we have preferred to confine our studies chiefly to skin reactions. This is, of course, not absolutely dependable, since responses of the skin need not necessarily run parallel to general hypersusceptibility. But accumulating clinical evidence seems to indicate that such a parallelism is fairly close, and if we remember possible divergences under special circumstances, this method need not lead us into error.

In choosing the proper animals for the experiments, the primary purpose of elucidating bacterial allergies as they occur in man naturally inclines one to choose that animal in which skin allergy is apt to be similar to that occurring in man. We have worked with guinea pigs as fulfilling this purpose more closely than most other animals, combining with this great convenience of experiment. But it is important to remember that with allergy as with true anaphylaxis, things which are true of guinea pigs are often quite inapplicable to other animals, notably rabbits. Reactions such as delayed bacterial allergy and the typical tuberculin reaction follow in guinea pigs very much the same rules, as to time and appearance, which are observed in man, though probably with considerably less quantitative delicacy. In favour of rabbits, of course, is the fact that the Arthus phenomenon which cannot easily or typically be elicited in guinea pigs occurs in rabbits, and we believe that in man the hastened reaction occurring upon subcutaneous injection of diphtheria antitoxin and the often disturbing reactions following the later injections of rabies vaccine are closely analogous to the Arthus phenomenon. On the whole, however, we believe that observations made upon guinea pigs by intracutaneous reactions will be most directly useful; and after this, it may perhaps be of some value to "cross-index" these facts with other animals.

In the observation of skin reactions of guinea pigs, there are many pitfalls which have led to a good deal of confusion to us and, we believe, to others, and before describing the experimental results it will pay to consider these, since no adequate observation can be made without taking them into consideration.

In the first place, it is necessary to evaluate the primary toxicity of the bacterial materials worked with, since thereby allergic skin effect becomes merely comparative by reason of the inflammatory reactions produced by the substance on the normal animal. In the second place, there are variations in individual guinea pigs in their reaction to bacterial materials, sometimes because of pregnancy, at other times perhaps because of infectious processes previously sustained. The most serious source of error is a non-specific hypersusceptibility which is not always, but usually developed in guinea pigs 8 to 14 days after the injection of massive amounts of any foreign protein. Such injections seem to induce a non-specific change of reaction capacity which gives rise to moderate but confusing inflammatory reactions when bacterial or other substances are subsequently

injected. We believe that many erroneous interpretations have resulted from this. Moreover, we believe that in itself this non-specific irritability of the skin, whatever its mechanism, following large parenteral injections of protein material may have considerable significance in skin reactions in general. Incidentally, the recent papers of E. F. Müller (9), on the profound influence of any intracutaneous injection upon the involuntary nervous system and the capillary mechanism, furnish a clue to the explanation of these occurrences.

For all these reasons we feel that it is of great importance to define the different types of skin responses to the injection of bacterial substances, although these have often been looked upon merely as quantitative differences in the intensity of one and the same mechanism. The following types of reaction may occur:

1. The purely toxic reaction which has no direct connection with allergy, and which is due to the primary toxicity of the bacterial materials. Examples of this are the Schick and Dick tests which may be indistinguishable from true allergic reactions.

2. The immediate urticarial skin reaction which appears within a short time is chiefly characterized by edema with little inflammation and fades promptly. This is the reaction which, in man, may be regarded as indicating protein sensitization, and which plays a minor rôle in some of the phenomena of bacterial allergy in guinea pigs.

3. The delayed skin reaction which usually does not become manifest in less than 5 or 6 hours, reaches its highest development within 18 to 48 hours, is characterized not only by edema, but by inflammatory changes, and which, by slowness of complete fading, indicates that more has occurred than a simple edema. These reactions are usually well demarked, like an erysipelas, but do not go on to central necrosis or the central hemorrhages. This is the common type of reaction obtained both in man and guinea pigs with bacterial products and, as we shall see, sometimes occurs with protein sensitization.

The classical tuberculin and abortin reactions probably consist, in part, of reactions of the preceding class but, superadded upon these, there is tissue injury with hemorrhage and sometimes necrosis. Although these reactions may be regarded as quantitatively intensified examples of the preceding, the frequency of severe central cell injury and necrotic changes in relatively small reactions of this type, as contrasted with the absence of these even in large reactions of the pre-

ceding class, suggests that there is in addition a factor involved in the hemorrhagic-necrotic ones which is absent in the former.

This classification is a tentative one, of course, since much about the mechanism of skin reactions is not yet clear, and we submit it as a working basis.

II.

Specificity of Bacterial Allergy.

It is, of course, of the greatest importance to determine from the beginning the degree to which the bacterial allergic phenomena with which we are dealing are specific. Specificity, apart from being a biological phenomenon of the greatest practical importance, furnishes a definite clue to mechanism, and much of the clinical experience of those engaged in doing skin reactions upon patients, as well as investigations in animals, has left much uncertainty regarding this question.

The following experiments, carried out with tubercle bacilli and various strains of *Bacillus abortus bovis* kindly furnished us by Dr. Theobald Smith, serve to define these relations.

The animals listed below were sensitized with living *Bacillus abortus* and subsequently tested by intracutaneous inoculations with O.T. and abortin produced from Bacillus 1211 by the method used to produce O.T.

1.	Living		*Bacillus abortus bovis*		Feb.	17.
2.	"	1211	"	"	Apr.	6.
3.	"	1207	"	"	"	19.
4.	"	1202	"	"	"	19.

Tested May 18.

	O.T. 1–20	Abortin 1–5
1.	+	++++ (slight necrosis)
2.	+	++++
3.	+	+++
4.	±	++

Since the O.T. in these tests had been recently made and had not been tested for potency, and the mild reactions with O.T. obtained above indicated a possibility of an insufficient dosage, these tests were repeated on the following day with an old lot of O.T. which had proved highly potent in 1–10 dilutions on many guinea pig tests.

On May 20 the animals were then again tested with this O.T. 1–10 and the same abortin 1–5 with the following results:

	O.T.	Abortin
1.	−	Severe ++++ with central white spot
2.	+	+++
3.	++	Strong +++
4.	+	+++

In these experiments it would appear that the specificity is a fairly strict one. As allergy becomes more extreme, however, the overlapping becomes more marked, as the following protocol of another set of animals done at a time when necrotic centres and hemorrhagic reactions were more violent illustrates.

	Abortin	O.T.
1. *B. abortus*-sensitized	+++	±
2. " " "	+	−
3. " " "	++++	+++
4. " " "	++	+++
5. Tuberculin-sensitized	±	+++
6. " "	−	++

In general it may be stated that *abortus* animals must remain in fairly good condition and resist infection without too rapid an emaciation. Otherwise they remain negative in the same way as a tuberculous animal in the prelethal stages. Given favourable conditions, however, they become positive about 8 days after infection.

The following experiment is added because it illustrates the same relative specificity with *Bacillus abortus* and staphylococcus animals, and further indicates the interesting fact which we consider of some importance, that with sufficient treatment animals may be rendered abortin-sensitive by injections of dead *Bacillus abortus* suspensions. This is again in contradiction of former opinions that only living bacilli can render such animals allergic, and in this way parallels the observations referred to above on analogous conditions with tuberculin sensitization. In this case the animals were prepared by 6 to 9 injections each of suspensions of *abortus* bacilli killed at 65° in the water bath. The tests here reported were done 3 weeks after the last injection.

Method of guinea pig preparation	Tested with abortin	Tested with staphylococcin
1. Dead *B. abortus*	Slight reaction	0
2. " " "	Large, hemorrhagic, severe	0
3. " " "	" not necrotic	0
4. " " "	Definite, not severe	0
5. Dead *Staphylococcus pyogenes aureus*	1 cm., redness, not raised	Slightly less than the abortin reaction on same pig
6. Dead *Staphylococcus pyogenes aureus*	0	0

Overlapping is further illustrated by a typhoid animal which had received 4 injections of formalinized typhoid bacilli intraperitoneally and 5 weeks after the last injection gave about equal reactions to typhoidin and 1–10 O.T. dilution, when before this it had given merely a weak typhoidin reaction and a negligible O.T. reaction.

It will be seen from these experiments, therefore, that there is a well defined specificity in bacterial allergy sufficiently definite to suggest an antigen-antibody mechanism; but that at the same time there is also a considerable amount of overlapping in animals that are highly sensitive, an overlapping not very different from that encountered in precipitation reactions with various types of bacterial nucleoproteins.

Further examples of specificity will be found recorded below in connection with skin tests on pneumococcus-sensitive animals.

III.

In preceding papers (10, 11) we have reported upon the production of allergic skin reactivity in guinea pigs treated with the formed cells and cell extracts of typhoid bacilli, staphylococci and streptococci. In all of these experiments the skin reactions obtained were of the delayed variety, appearing from 12 to 24 hours after intracutaneous injection, and manifested by well outlined reddened areas, slightly edematous and swollen—never, however, even in the most marked cases, showing the central necrosis and hemorrhage characterizing the most severe forms of tuberculin and abortin reactions.

Our experiments with Grinnell upon streptococci which, in harmony with the observations of Dochez and Sherman (12), showed that it is

relatively easy to sensitize to streptococci by the repeated intraperitoneal administration of dead or of living bacteria; that the skin reactions, best elicited with the Dick filtrates, become positive 2 to 4 weeks after the last injection; and that continued immunization causes this hypersensitiveness to fade. This point, again, is corroborative of Dochez and Sherman's observation on the neutralization *in vitro* of the allergic antigen by antistreptococcus serum, and of the observations of Mackenzie and Woo (13) on a similar fading of analogous sensitiveness induced with pneumococcus extracts.

In later work we extended our observations to allergy induced with a number of bacteria other than the tubercle bacillus, including the pneumococcus, staphylococcus, typhoid bacillus and *B. abortus bovis*. With all of these bacteria it was possible to render animals allergic in the same manner in which this was accomplished with streptococci. In considering the mechanism of these reactions, we were led to undertake investigations concerning the differences in the types of antibodies developed in animals by the injection of the several fractions of the bacterial cell, the results of which we published (14) simultaneously with similar studies by Avery and Heidelberger (15).

This work revealed relations which could not fail to have important bearing upon allergic reactions, if these were in any way dependent upon antigen-antibody combinations. Summarized briefly, it was shown that there was a fundamental difference in the nature of antibody production determined by the form in which the bacterial antigen was injected and upon the particular fraction of the bacterial extracts employed. To illustrate with the pneumococcus, which is the easiest organism to work with in this respect, the facts are as follows:

If a rabbit is immunized with whole pneumococci, the antibodies formed react with whole bacteria, agglutinating them in the usual way, and the same serum will precipitate the carbohydrate fraction or residue (soluble substance of Avery and Heidelberger). Since it is practically impossible to prevent some pneumococci from going into solution, even when formalin is applied directly to the young growths, such serum will also react with nucleoprotein.

If a rabbit is immunized either with pneumococci dissolved in bile and filtered,

or with the so called "nucleoprotein fraction," the antibodies formed react with such nucleoprotein, but hardly at all with the residue material mentioned above, and contain little or none of the ordinary antibodies that act upon the whole formed cells. Immunization with the residue fraction alone of course produces no antibodies whatever. The ordinary antipneumococcus sera put out for therapeutic and diagnostic purposes contain antibodies for both types. This must be borne in mind in subsequent considerations.

In taking account of those relations, then, it became necessary to sensitize in such a manner that both types of antibody would be likely to be formed and to do subsequent tests upon the skin with the materials specifically reacting with the respective antibodies. The pneumococcus offered the most convenient material for this purpose, largely because it has been found possible to destroy the complex antigen responsible for the formation of the anti-residue (anti-"whole" pneumococcus) antibodies by dissolving the bacteria in bile.

We proceeded first, therefore, to endeavour to define the relations of the carbohydrate residue antigen to the allergic reaction, as follows:

Active and Passive Sensitization to the Carbohydrate Residue (Soluble Specific Substance).

Our attempts to produce typical delayed skin reactions in animals with the carbohydrate, type-specific residue antigens were so uniformly negative that they may be very briefly summarized.

Whatever the method of active sensitization, subsequent skin tests with the homologous type-specific carbohydrate residue never produced typical delayed allergic reactions. Occasionally the residue injection gave rise to moderate immediate edematous swellings which disappeared within a short time and were usually gone completely on the following day, when typical reactions are most pronounced.

The same is true of animals treated with homologous sera potent with antibodies which specifically precipitated the residue *in vitro*. A great many attempts were made to sensitize passively in this manner and large amounts of serum were given in single and multiple injections; reactions were attempted at intervals ranging from a few hours after the injection of the serum up to several weeks. But the results remained negative and as described above.

The intracutaneous injection of formed precipitates resulting from the incubation of mixtures of residue and antiserum never produced typical skin reactions. The supernatant fluids of such mixtures were likewise negative.

From many experiments of this nature, together with the corroborative evidence of our other work, we feel confident in asserting that the reaction which is represented by the specific union of the type-specific residue or soluble specific substance of the bacteria (the haptophore group of the bacterial antigen) and their homologous antibodies (representing the agglutinins, precipitins, etc., of antibacterial sera) has absolutely no relationship to that form of bacterial allergy which is manifested by the delayed skin reaction. This, incidentally, is consistent with the results of Mackenzie and Woo, who found that no relationship existed between allergy and that protective mechanism which is represented by such antibodies.

The Relationship of the Bacterial Nucleoprotein and Its Antibodies to Allergic Reactions.

Having failed to demonstrate any relationship between the isolated residue antigen and the bacterial antibodies to the allergic reaction, we next proceeded to carry out skin reactions with bacterial substances so prepared that either the total suspended materials or their nucleoprotein constituents were represented in the test material. The test on page 763 is an experiment of this kind with pneumococcus.

If the following protocol is examined from the point of view of possible relationship between allergy and antinucleoprotein antibodies, it will be seen that skin reactions were obtained with bile solutions of the pneumococcus in animals in which residue reactions were negative, and that such reactions were best developed in animals treated with these same bile solutions. It stands to reason that the cited experiments represent only a few of a considerably larger number and the results such as those reported tend to indicate a possible relationship between nucleoprotein and its antibodies in the allergic phenomena.

Guinea Pig 1 (845 gm.). Received 2 cc. of filtered bile solution of pneumococcus
Guinea Pig 2 (670 gm.). on Mar. 23, 24, 25, 26 and 27, intraperitoneally.

Guinea Pig 3 (670 gm.). Received 2 cc. of 70°C. killed fresh suspensions of whole
Guinea Pig 4 (620 gm.). pneumococci on Mar. 23, 24, 25, 26 and 27. Considerable loss of weight.

Intracutaneous Tests.

	Apr. 6		Apr. 10	Apr. 12		Apr. 13		Apr. 20	
	Skin test with bile solution	Skin test with whole pneumococci		Residue	Bile pneumococci	Pure bile 1–10*	Bile pneumococci	Residue	Bile pneumococci
1. Treated with bile solution	±	+	Reinjected intraperitoneally with 2 cc. bile solution	−†	+++	±	+++	−	−
2. Treated with bile solution	+++	−		−	+++	±	++	−	++
3. Treated with whole pneumococci	+	−	Reinjected with whole, dead pneumococci	−	+	−	+	−	−
4. Treated with whole pneumococci	±	−		−	+	±	++	−	−

* These tests are a few of many that have been done with each preparation to check up the non-toxic action of remnants of bile. Our bile solutions of pneumococcus were all made by adding a minimum amount of ox bile for complete solution which ranged from concentrations of 1–15 to 1–10 with the suspensions used. It was impossible to separate the bile from the nucleoprotein by precipitation because such precipitation brings down the bile salts. These were gotten rid of in most of the preparations used, if not completely at least to a large extent, by 48 hours' dialyzing against salt solution.

† These residue reactions check up information previously obtained that the residue antigen gives no skin reaction in guinea pigs that are sensitized to the nucleoprotein, as shown by the bile solution reaction obtained on the same day. Again, there is no residue skin reaction in Animals 3 and 4, although, being treated with whole pneumococci, there must have been a formation of antibodies capable of reacting with the residue.

An experiment with tubercle bacillus nucleoprotein is illustrated in the following:

The nucleoprotein extract was filtered through Berkefeld candles in order to remove possibility of injecting dead bacilli, or fragments.

The injections were made as follows:

Guinea Pig 1 received 6 injections ranging from 1 to 5 cc. at 4 day intervals between Jan. 13 and Feb. 3.

Guinea Pig 2 received 5 similar injections ranging from 5 to 7 cc. between Jan. 21 and Feb. 7.

Intracutaneous tests with nucleoprotein solutions and O.T. 1–5 were begun on these animals on Feb. 5 and carried out as follows:

Guinea pig	Feb. 5		Feb. 8		Feb. 10		Feb. 18		Feb. 19		Feb. 25	
	O.T.	N.P.	O.T.	N.P.	O.T.	N.P.	O.T.	N.P.	O.T.	N.P.	O.T.	N.P.
1	−	−	−	−	+	−	++	Not	++	+	−	−
2	−	−	±	−	±	±	++	done	+	+	+	+

Reactions in such an experiment are always better with O.T. than with pure nucleoprotein, a fact which we attribute to a possible denaturization of the nucleoprotein in production, since not only in these experiments but in all experiments such relatively purified nucleoprotein is a very poor antigen and must be injected in large quantities and in many doses.

In all experiments of this nature there is an eventual tendency to desensitization, that is, a fading of the reactions upon continuous treatment, an observation which is in agreement with those of Mackenzie and Woo. Apart from the practical significance of this, it is an immunological fact which tends further to arouse suspicion that such sensitizations may, to some extent, be dependent upon an antigen-antibody mechanism.

The fact that guinea pigs sensitized with tubercle bacillus nucleoprotein reacted well with O.T. suggested another attempt to sensitize with O.T. direct. This has not been noticed either by others or ourselves in the past as a result of repeated skin reaction, but when, in analogy with nucleoprotein sensitization, we injected guinea pigs with relatively large amounts of O.T.—that is, 3 to 6 injections of about 0.3 cc. of concentrated O.T. in proper dilution, intraperitoneally,

filtered through Berkefeld filters to prevent the introduction of bacilli—we found that a sensitization could be obtained which was analogous to that with nucleoprotein; indeed, the animals reacted with large, flat, reddened areas, usually of considerably greater dimensions than those sensitized with nucleoprotein.

Experiments on Passive Sensitization to the Bacterial Nucleoprotein Fraction.

We will not go into detail concerning these experiments because the technique is simple and the results inconclusive. However, when a rabbit was treated with filtered nucleoproteins from the tubercle bacillus for a long time and with large quantities and the serum so obtained was found to precipitate tubercle bacillus nucleoprotein, normal guinea pigs were intraperitoneally injected with amounts ranging from 5 to 8 cc. and skin tests performed on them both with O.T. and nucleoprotein solutions every other day for some time after the serum administration.

It was found that in many of these pigs a definite allergic response was obtained both to O.T. 1–10 and to nucleoprotein solutions. These reactions did not, however, appear sooner than 6, 7 or 8 days after the serum administration, and usually faded within 3 weeks.

Similar experiments were done rather more extensively with pneumococcus substances in which we injected guinea pigs not only with the sera of rabbits immunized with bile extracts, but with the ordinary antipneumococcus Type I therapeutic sera which possessed a not inconsiderable capacity for precipitating nucleoproteins and bile solutions.

We do not cite protocols of these experiments because, carefully analyzed, they did not furnish conclusive evidence of passive sensitization. Although indicating the likelihood of such a process in many instances the results were complicated by the occasional development of similar sensitiveness in control animals prepared with normal horse serum. Moreover the consistent lateness of the appearance of sensitiveness whenever it appeared, indicated that the mere introduction of the antibodies could not be regarded as explaining the phenomenon as a whole.

We are confronted with the curious contradiction, therefore, that

while the facts of active sensitization, specificity and desensitization, can be explained only on the basis of some mechanism analogous to that of antibodies, the passive experiment remains unconvincing. This may be interpreted as meaning either that we are dealing with a type of reaction in which antibodies, in the ordinary sense of the word, play no rôle whatever; or that such antibodies are indeed significant but represent only that part of the mechanism which determines specificity and desensitization, another additional factor being required to complete the reaction. It is this latter interpretation which seems to us the more likely one for reasons that will be elucidated in the following section in which we deal with the tuberculin reaction itself.[1]

IV.

The Tuberculin Reaction Itself.

The preceding experiments may be regarded purely as preliminary to a study of the tuberculin reaction itself in that we believe that they have completely eliminated the possibility of a residue antibody mechanism in the tuberculin reaction, but have shown that a certain amount of sensitization can be obtained by active treatment with nucleoproteins. They have also again emphasized the existence of a specific element in these reactions, facts which to some extent clear up the underbrush but still leave us in the dark concerning the complete mechanism of the reactions.

Another series of preliminary experiments carried out during the past year too voluminous to be reported in detail, but necessary because of the many contradictions in the literature, may be briefly summarized as yielding the following information: (1) that contact *in vitro* of O.T. with the serum of animals immunized with living or dead tubercle bacilli does not produce anything which will give tuberculin

[1] We have so far failed also in obtaining any *in vitro* or *in vivo* neutralization of the allergic substance either by mixing with various antisera and incubating or by preceding skin tests by intravenous serum injections. To this we attribute less importance, however, since we bear in mind the great difficulty that Weil (16) and others have experienced in similar attempts with protein anaphylaxis where the antigen-antibody relations are far more clear. It seems that in all forms of sensitization it is not easy to divert antigen from sensitive cells, even with high concentrations of circulating antibodies.

reactions in the normal animal, whether or not complement be present; (2) that the same is true when the serum of animals immunized with tubercle bacillus nucleoprotein is employed; (3) that precipitates formed in both types of reaction fail to produce tuberculin reactions in normal guinea pigs, even when treated with complement; and (4) that none of the sera mentioned above will neutralize the action of O.T. upon tuberculous guinea pigs.

These facts in general correspond quite closely with those ascertained for bacterial allergy with other organisms. A direction of experimentation which was now indicated was that which dealt particularly with the meaning of the inflammatory tissue reactions, the tubercles, which may be recognized as possessing considerable significance in the development of tuberculin hypersensitiveness. In a former paper we have already shown that a certain amount of passive sensitization can be obtained by the injection into guinea pigs of sera from rabbits in which a large amount of tuberculous inflammatory reaction has been incited by the establishment of multiple tubercles. We were also able at that time to confirm the observation of Lange (17) that some sensitization can be incited by repeated injection of tuberculous tissue filtrates. In none of this work, however, did the sensitization amount to very much more than that which we have more recently obtained with nucleoproteins. Never was there any indication of hemorrhage or necrosis. These experiments have been repeated with the same results.

We therefore investigated the McJunkin (18) experiment in an effort to procure from it a possible clue, since we have been able in the past to confirm this procedure by the method originally reported by him. In attempting to analyze this method we determined the following facts:

1. A normal guinea pig injected with a filtered exudate from the peritoneum of a tuberculous guinea pig killed by intraperitoneal injection of O.T., gives an excellent skin reaction 11 to 12 days after the administration of the filtrate.

2. Similar exudates obtained by injecting a tuberculous guinea pig with broth sensitized definitely but less strongly than in the first case, also in about 11 days.

3. Exudates obtained from normal guinea pigs with broth gave a very slight and probably non-specific sensitization to O.T. in 11 days to 2 weeks.

These experiments confirmed McJunkin's original claims, but did not shed any particular light on the mechanism of the reaction. Indeed, while it seems, as McJunkin supposed in his first report, that we are confronted with an active sensitization of some kind, this is not at all certain.

We now proceeded, therefore, to investigate the possibility that in the reaction between the tuberculous tissues and products of the tubercle bacillus there might be formed a toxic substance responsible for the reaction. The hope of obtaining some light in this direction was encouraged by two observations. One of these was an observation made with pneumococcus at about the same time that Julianelle and Reimann (19) published their observations upon the purpura-producing, autolytic substance derived from pneumococci. The observation was as follows:

If a freshly prepared pneumococcus suspension is divided into three parts, one of them left undisturbed except for the addition of a minute amount of thymol, the second immediately heated to 65° for 15 minutes and the third dissolved in bile, a similar amount of thymol added to the last two to equalize conditions and all three set into the incubator and allowed to stay there for 48 to 72 hours, and skin reactions carried out with 0.2 cc. of each of these suspensions in a normal guinea pig, there will be very feeble, or practically no reactions over the bile solution area or that carried out with the pneumococci immediately heated, but over the area into which the autolyzed pneumococci were injected there will be formed a violent delayed reaction which in all its morphological and pathological features resembles a violent, severe tuberculin reaction in a tuberculous guinea pig injected with O.T. These relations are shown in the accompanying figure.

Similar experiments carried out with meningococcus show comparable conditions, except that in meningococcus the immediately heated organisms often showed a reaction similar to but milder than that of the autolyzed ones.

With *Bacillus abortus bovis* an analogous experiment can be performed, except that here autolysis seems to be extremely slow and reactions comparable to the allergic ones do not develop until the unheated cultures have been kept in the incubator for 8 or 9 days and then they are not as violent as those produced with pneumococcus.

These experiments indicated the possibility that bacterial products may yield, upon cleavage, a substance toxic for the normal animal as O.T. is for the tuberculous animal.[2]

It was obviously suggested, therefore, again to investigate the results of the incubation of mixtures of O.T. with the extracts of tuberculous tissues.

This thought was further encouraged by observations that had been made in connection with earlier attempts to neutralize O.T. for tuberculous animals by similar incubation. An experiment of this type is as follows:

A mixture was made of one part of O.T. with five parts of a clear, filtered extract made by macerating and shaking the skin of a tuberculous animal in slightly alkaline salt solution. The mixture was allowed to stand for several hours in an incubator and overnight in the ice box.

0.1 cc. of this was then injected into a tuberculous animal, and in another spot a fresh 1–5 solution of the same tuberculin in salt solution. As shown in the figure, in this particular experiment the reaction over the point of injection of the mixture was markedly larger than that which developed where the fresh O.T. dilution had been injected.

Conditions similar to the above were encountered on a number of occasions, usually when extracts of lung or skin were used, less frequently when the tuberculous tissue extract was furnished by spleen or liver tissue.

From these two types of experiment it seems quite likely that in addition to the ordinary antigen-antibody reaction, tuberculin allergy was in some way related to a direct reaction between the inflammatory tissue reactions and the tuberculin, a thought which, incidentally, is suggested by all the past history of studies of tuberculin reactions.

Experiments on the Action of Tuberculous Tissue upon O.T.

This line of investigation was followed for a very long time with tantalizingly encouraging, but inconclusive results. We were never

[2] We assume that this toxic substance is the same as the purpuric poison described by Julianelle and Reimann, since it is produced in more or less the same way and since it possesses a considerable heat stability. The powerful toxicity of this material for guinea pigs, as shown in the figure, opens the question of its relationship to other described pneumococcus poisons and necessitates the further investigation of its possible antigenic properties, a matter that is being worked upon.

satisfied that we were obtaining anything more than definite suggestions in a positive direction until we abandoned the efforts with tissue extracts and began to work with macerated cellular materials, unfiltered. The observation which most encouraged us to continue in this direction was the fact that we again and again noticed that mixtures of O.T. with tissue extracts, even though they had relatively little action upon a normal animal, were considerably more toxic than O.T. alone for the tuberculous animal.

We do not cite a large number of unsuccessful experiments, but restrict ourselves to typical examples of the final experiments which convinced us that the interaction between O.T. and tuberculous tissue played an important rôle in the mechanism of tuberculin reactions. The following is such an example.

Mixtures were made as follows: Lung tissue of a tuberculous guinea pig was macerated in a mortar with sterile sand, and small amounts of salt solution added. A similar maceration of normal lung was made at the same time. The following preparations were then set up in test-tubes.

1. Macerated bits of lung in salt solution, 5 cc., + 0.1 cc. of concentrated O.T.
2. Similar tuberculous lung without O.T.
3. Salt solution, 5 cc., + concentrated tuberculin, 0.1 cc.
4. Normal lung, 5 cc., + O.T., 0.1 cc.

To each one of these tubes a small bit of thymol was added in order to prevent putrefaction, and the tubes were incubated. After 24 hours small amounts of fluid were taken from each of these tubes, centrifuged at high speed and 0.2 cc. respectively injected into a large white normal guinea pig.

The results are shown in Fig. 5 and are self-explanatory. It will be seen that while the normal lung macerate and the O.T. alone produced slight reactions, the incubated tuberculous lung macerate with O.T. gave a strong and extensive reaction.

Another experiment of the same type done with human lung is the following:

Normal human lung and lung thickly studded with miliary tubercles were obtained through the kindness of our hospital associates. Pieces of each were cut up with a pair of scissors and macerated with sand in a mortar, salt solution being added. Finally the sand was allowed to settle and the supernatant suspension containing bits of lung was taken up into tubes as follows:

1. Normal human lung suspension, 10 cc.
2. Normal human lung suspension, 10 cc., + O.T., 0.3 cc.
3. Tuberculous lung suspension, 10 cc.
4. Tuberculous lung suspension, 10 cc., + O.T., 0.3 cc.

Small, approximately equal pieces of thymol were added to each tube. After 48 hours' incubation portions of each tube were taken out, centrifugalized at high speed and 0.2 cc. injected intracutaneously into a normal, large, white guinea pig.

The results are shown in Fig. 6. Again the area into which supernatant fluid from Tube 4, containing the tuberculous lung suspension with O.T., had been injected gave a reaction larger than that given by the material from Tube 3, and both of these were much more extensive than those from the two controls.

We assume that the results in Tube 3 of both experiments were due to the presence of a certain amount of tubercle bacillus material in the infected tissue.

SUMMARY.

Our experiments have confirmed the fact that the so called bacterial allergies are dependent upon a mechanism which differs materially from that determining true protein anaphylaxis. Anaphylaxis to protein substances of the bacteria probably occurs but plays a relatively unimportant rôle in the phenomena of infection. The bacterial allergies, however, are of great importance since they develop rapidly and render the infected animal highly vulnerable to products of the bacterial growth which are relatively innocuous for the normal animal.

Neither the type-specific carbohydrate "residue antigens" (the "soluble specific substances" of Avery and Heidelberger) nor the antibodies reacting with them play any part whatever in bacterial allergy, and since these type-specific substances represent the haptophore groups of the whole bacteria by which they react with the agglutinins, precipitins, sensitizers, etc., of immune serum, allergy, as previously determined by Mackenzie and Woo, is in no way related to that phase of resistance which is determined by these antibodies. This does not, however, preclude the possibility that allergic hypersusceptibility may not in some way be related to other factors of resistance more definitely associated with cellular rather than with intravascular reactions. Our previous studies with Jennings and Ward in tuberculosis point in this direction (20).

Guinea pigs can be actively sensitized with all the bacteria with which we have worked when repeated injections of whole bacteria or of the protein (nucleoprotein) fraction are administered. Large amounts of the latter are necessary since these materials are indifferent antigens, possibly because of the severe manipulations necessary in their production.

Sensitiveness develops usually within 10 days after the first dose and increases with continued treatment for 3 or 4 weeks.

Sensitiveness is relatively specific, by which we mean that there is a definite specificity which, however, in highly sensitive animals is not absolute and shows considerable overlapping.

Continued treatment with considerable quantities of the above substances leads to gradual desensitization in animals in which there are no chronic foci present, which, as in tuberculosis, tends to continue the sensitization.

Attempts at passive sensitization have been irregular and inconclusive. When any degree of sensitiveness has developed after the injection of immune sera, it has appeared late and has been of doubtful specificity. Conversely we have failed in any case to neutralize the activity of the active allergic constituents of bacterial extracts by incubation with any type of immune serum.

We have failed so far to show any increased fixation of tuberculin material on the part of tuberculous tissues or on that of living tuberculous animals. These failures, however, seem to us of relatively slight importance since quantitative experiments of this nature are extremely difficult in the case of a substance as delicately potent for the tuberculous animal.

On the other hand we have obtained definite, though irregular evidence that the incubation of O.T. with fragments of tuberculous lung tissue (less clearly with other tissues) leads to the formation of a substance that produces allergy-like lesions in the skin of normal guinea pigs. With somewhat greater regularity, similar treatment of O.T. has enhanced the potency of the tuberculin for tuberculous animals. And, in these experiments there was evidence that the factor responsible for this action was not easily separable from the cells themselves.[3]

When these experimental data are analytically considered they appear in many respects confusing and contradictory. There has been so much work done on the tuberculin reaction, moreover, that, in the face of experimental inconsistencies it would seem foolhardy to formulate more than tentative suggestions to explain the mechanism

[3] We are permitted by Dr. Petroff of Saranac to state that he has obtained results similar in principle with our own, but also irregular and not repeatable at will

of these reactions. Nevertheless there are a few outstanding and sufficiently reliable facts which compel a limited number of definite deductions.

In the first place there is no question of the complete independence of the true allergic phenomena from the ordinary bacterial antigen-antibody reactions. We know, moreover, that the allergic substance is chemically separable from the carbohydrate "residue" or haptophore group of the bacteria (Mueller, Laidlaw and Dudley). Indeed it has been shown by Long and Seibert (21) that the active allergic substance is either a protein in itself, or at any rate closely associated with the bacterial protein.

Furthermore, the distinct, though limited, specificity of the allergic sensitiveness compels the conclusion that we are dealing with an immunological process in which the tissue cells acquire an increased specific capacity to react with this nitrogenous material, a capacity which, in principle, is not far removed from the supposed "sessile receptor" apparatus which is conventionally held responsible for protein anaphylaxis; and this analogy is further amplified by the apparent desensitization which continued treatment produced in many of our own experiments as well as in those of Mackenzie and Woo.

Here, however, the analogy with protein anaphylaxis ends. Passive sensitization with any form of immune serum or with the sera of highly sensitized animals is either feeble or entirely unsuccessful and indicates quite convincingly that, whatever the receptor apparatus of the cells may be, it is not easily given up to the blood stream as are ordinary antibodies. Further than this, our tissue-tuberculin experiments, irregular and occasional as they were, nevertheless convinced us that:

1. The contact with the tissues of tuberculous animals results in the production of a toxic factor, not unlike the autolytic toxic materials of some bacteria.

2. The active cell constituent by which this action is wrought, is not easily separated from the cells, even by energetic methods of extraction.

This close association of the entire process with the cells themselves is particularly significant in view of the obvious cell injury in which these delayed allergic effects differ from the ordinary urticarial, evanescent reactions associated with protein anaphylaxis.

The process of allergy, as far as we can approach it then, may be conceived as follows:

A nitrogenous, probably protein, constituent of the bacterial growth or of its body substance stimulates a specific reaction in the tissue cell by which its specific capacity to establish contact with this constituent is enhanced.

The cell is thereby enabled to exert a, probably, enzyme-like effect upon this material in consequence of which a toxic substance is liberated, largely upon or possibly within the cell itself.

Both processes may be dependent upon one and the same reaction body. But it seems more likely that increased contact and the increased cell activity are separately developed, an assumption which is rendered probable by the association of the highest degrees of allergy with inflammatory cell reactions, and by the fact that moderate and less specific allergic sensitiveness follows 10 or more days after the administration of considerable amounts of indifferent protein substances to guinea pigs. We interpret this as signifying that such injections may non-specifically increase cellular activity, a change which many earlier workers have spoken of as "cell irritability."

Both processes are closely associated with the altered cell itself and the factors by which the reaction is brought about are not easily given up to the blood stream as are the antibodies formed in response to injections of proteins or whole bacteria.

We are confronted, therefore, with an immunological mechanism which has some close analogies to those others in which circulating antibodies are formed, but which differs from these mainly in the intimacy with which the entire reacting system is associated with the cells themselves.

It is difficult to conceive that a functional cell alteration, as profound as this, should be entirely unrelated to the phenomena of susceptibility or resistance.

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EXPLANATION OF PLATES.

PLATE 29.

FIG. 1. Severe type of skin reaction given in guinea pigs sensitized by active immunization with nucleoprotein. This particular guinea pig happens to have been one sensitized by the McJunkin method with the exudate produced in a tuberculous guinea pig with O.T., but the type of the reaction represents the average severity of the non-necrotic delayed skin reactions referred to in the text.

FIG. 2. Comparative reactions in an *abortus*-sensitized pig during the early stages of severe reaction with (1) 1–10 tuberculin and (2) 1–5 abortin.

FIG. 3. Typical severe skin reaction on a normal guinea pig obtained by the injection of 0.2 cc. of pneumococci autolyzed in salt solution for 48 hours.

PLATE 30.

FIG. 4. Tuberculous guinea pig tested over Area 1 with O.T. 1–5, over Area 2 with O.T. diluted to the same extent with concentrated extract of the skin of a tuberculous guinea pig and kept in the incubator for 4 hours before the two tests were done. Note the much larger extent of the area over 2.

FIG. 5. The figures in this illustration show the resulting inflammatory areas on the skin of a normal guinea pig injected intracutaneously as follows:

1. Supernatant fluid from a 2 days' incubation of fragments of normal lung tissue with O.T. in a concentration of 1–33.
2. O.T. in solution 1–33, similarly incubated.
3. Tuberculous human lung fragments in salt solution.

4. Tuberculous human lung fragments + O.T. in a concentration similar to the above.

Small pieces of thymol had been added to all the preparations to prevent infection with bacteria.

Small amounts of fluid were removed with a pipette and centrifuga'ized at high speed, the clear supernatant fluid being used for intracutaneous injection.

FIG. 6. Comparative areas of inflammatory edema on the skin of a normal guinea pig resulting from intracutaneous injections with the following materials:

1. Normal lung fragments + O.T., total concentration 1–10.
2. O.T. 1–10 in salt solution.
3. Tuberculous lung without O.T.
4. Tuberculous lung with O.T.

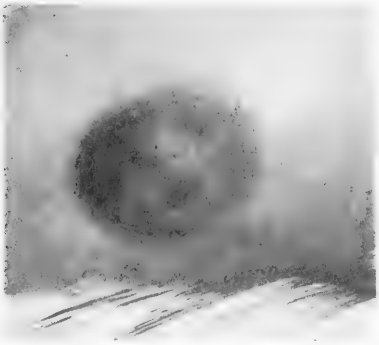

FIG. 1.

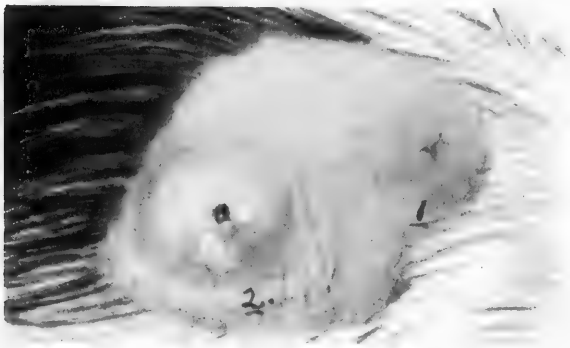

FIG. 2.

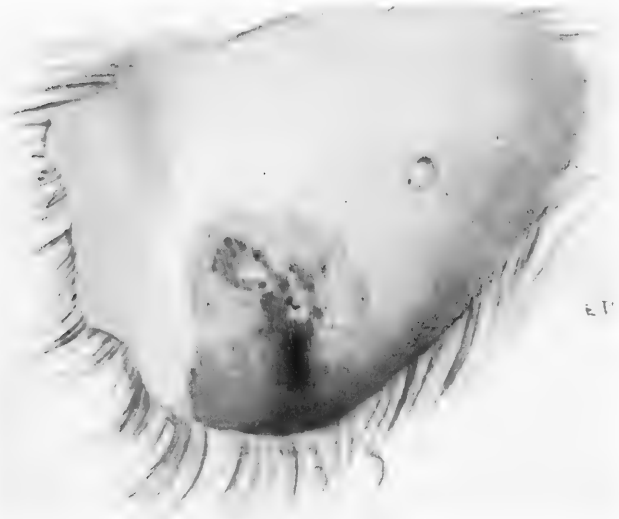

FIG. 3.

(Zinsser and Tamiya: Analysis of bacterial allergy.)

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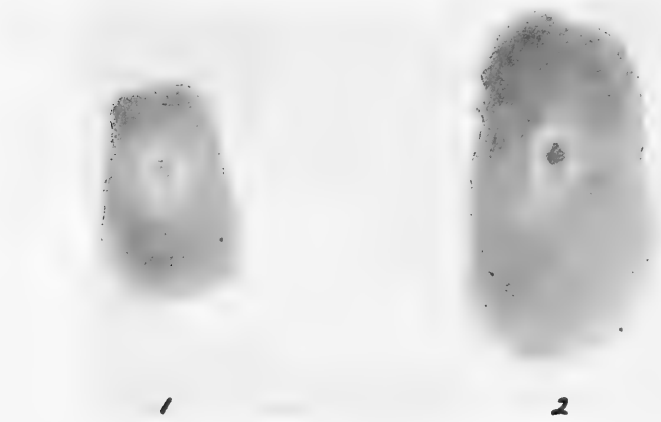

FIG. 4.

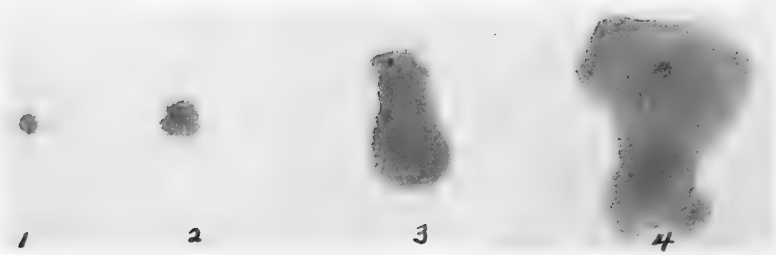

FIG. 5.

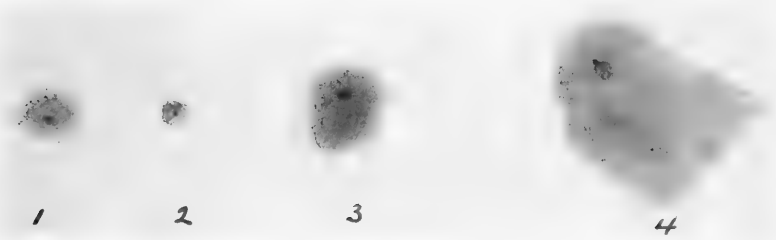

FIG. 6.

(Zinsser and Tamiya: Analysis of bacterial allergy.)

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TISSUE-DIGESTING ENZYME (HISTASE) OF STREPTOCOCCI.

By MARTIN FROBISHER, Jr., Sc.D.

(From the Department of Pathology and Bacteriology of the Johns Hopkins University, Baltimore.)

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In the fall of 1925, while making a study of beta type hemolytic streptococci from a variety of sources, a strain was isolated from impetigo-like lesions which was found to be capable of digesting a considerable portion of the meat in tubes of Holman's (1) modification of Robertson's cooked meat medium. It did not liquefy gelatin and in every other respect resembled *S. pyogenes*.

Digestion of animal tissues by streptococci of the type represented by *S. pyogenes* has not been described before, as far as can be ascertained from a search of the available literature. Digestion of casein has been observed and reference to this will be made later on.

Description of the Tissue Digestion.

Tubes of cooked meat medium with vaseline seals were inoculated with the proteolytic strain of streptococcus referred to above. The level of the chopped meat in the tubes was carefully marked with a red wax pencil. After 24 hours at 37°C. no change in the level of the meat had occurred. After 48 hours, however, the level slowly descended. Smears made at this time showed pure cultures of the streptococci. After 5 days at 37°C. about 30 per cent of the meat had completely disappeared. The meat continued to be liquefied during 3 subsequent weeks at room temperature. At the end of 1 month about 75 per cent of the meat had disappeared and the remainder had the finely granular appearance sometimes seen in cooked meat cultures of very proteolytic anaerobic bacilli. No gas or foul odor was produced.

Occurrence in Filtrates.

Several tubes of cooked meat were inoculated and allowed to incubate for 5 days at 37°C. Part of the tissue having liquefied, the supernatant fluid was removed and passed through a Berkefeld filter. The filtrate was incubated along with subcultures from it. After 2 weeks both were found sterile.

The level of the meat in three tubes of cooked meat medium was carefully marked as above. The first tube received 1 cc. of the sterile filtrate. The second received 0.5 cc. and the third tube was not opened. All three were incubated at 37°C. After 18 hours the meat level in the two tubes containing the filtrate was about 2 mm. below the mark. The control tube was unchanged. After 5 days the meat level in the filtrate-containing tubes was about 8 mm. below the mark while the control was still unchanged. It appears that a true ectoenzyme is responsible for the tissue digestion. The name histase is proposed for this enzyme.

Effect of Heat on the Enzyme.

The meat level in six tubes of cooked meat medium was carefully marked. To the first was added 1 cc. of the sterile filtrate described above. A tube containing 5 cc. of the filtrate was then immersed in a water bath held at 60°C. After waiting 4 minutes for the temperature to become uniform, 1 cc. of the filtrate was removed at 15 minute intervals and placed in one of the marked tubes of cooked meat. All tubes were then incubated at 37°C. with an unopened control and a sterility control. Tissue in all tubes containing filtrate was digested, but not at the same rate. After 5 days visible digestion had taken place at about equal rates in tubes heated not longer than 30 minutes. About 2 days later, liquefaction was observed in the 45 minute tube and about 3 days subsequent to this, digestion was discernible in the tube heated for 1 hour. After 2 weeks all tubes showed about the same degree of digestion. Exposure to 60°C. for 1 hour, therefore, seems to retard the action of the enzyme but not completely to destroy it. It may be that most of the enzyme was destroyed but that sufficient remained to digest a few mm. of the meat.

Action of the Enzyme on Human Tissues.

In preparing the cooked meat medium referred to above, the method of Holman (1) was adopted, with the exception that beef hearts were used and vaseline seals were added to the tubes. Similar tubes were prepared with human instead of bovine heart. The human heart was taken from an uninfected case at autopsy. Nine strains of hemolytic streptococci were studied in this medium. Of these, three did not digest bovine or human heart, while six digested both.

In several tubes inoculated with streptococci and sealed with vaseline, digestion failed to occur. Smears were made to determine whether growth had occurred. It was found to be so sparse as to leave the ability of the organisms to digest meat in doubt. The vaseline seals were removed with sterile pipettes and the cultures reincubated. After 24 hours good growth had occurred and after 5 days extensive digestion was observed. These strains were evidently fairly strict aerobes. This peculiarity has since been observed in several other cases. A difference in the appearance of the meat in tubes treated in this manner deserves mention. In such tubes, after growth has occurred, the color of the meat changes from the usual red-brown to a greenish grey most intense at the top surface. The meat in which growth has occurred without the removal of the vaseline seal does not change color, or at most becomes a slightly darker brown. Strains which digest meat in the presence of the vaseline seal, do not produce any color change when cultivated in tubes from which the seal has been removed, although they still digest the meat.

Relation to Hemolysin.

Julianelle (2), in a study of the hemolytic staphylococci, obtained data suggesting that proteolysis and hemolysis by those organisms are somewhat closely related. With this in mind twenty-two strains of hemolytic streptococci from various sources were tested for ability to digest meat and to produce hemolysin. For the hemolysin test the organisms were cultivated for 18 hours in infusion bouillon at 37°C. In each test 0.5 cc. of culture was mixed, with 0.5 cc. of 5 per cent suspension of washed rabbit corpuscles. The mixtures were shaken and placed in the water bath at 37°C. Readings were made at the

end of 90 minutes. The results of the comparisons are shown in Table I. This series would seem to indicate that proteolysis and hemolysis by this type of organism are not closely related. It is

TABLE I.

Relation between Hemolysis and Proteolysis.

Strain No.	Source	Tube hemolysis* (90 min.)	Meat digestion
8556	Septicemia	4+	−
3606	Certified milk	−	−
Reed.	Infected ear	4+	−
8674	Septicemia	−	+
Mort. 23	Septic sore throat	4+	+
Lexley	Infected ear	4+	+
X 40	Septic sore throat	4+	+
8616	Erysipelas	4+	+
S 2	Scarlet fever	4+	+
X 32	Septic sore throat	2+	+
X 39	Septic sore throat	4+	−
X 41	Septic sore throat	4+	+
Cow 108	Mastitis	4+	+
3641	Certified milk	+	±
Hamer	Sore throat	4+	+
2735	Certified milk	4+	+
8576	Septicemia	+	±
2082	Certified milk	4+	+
3639	Certified milk	+	−
2081	Certified milk	4+	−
Hebron	Sore throat	4+	+
2600	Certified milk	4+	−

*4+ indicates complete hemolysis.

Summary:

Strains digesting meat and producing soluble hemolysin	12
Strains digesting meat and not producing soluble hemolysin	1
Strains not digesting meat but producing soluble hemolysin	6
Strains not digesting meat and not producing soluble hemolysin	1
Doubtful	2

possible that the method used for determination of hemolysin production is too crude. De Kruif and Ireland (3) have found that hemoly-

sin may disappear from cultures more than 14 hours old. It is evident however that some strains of streptococci may produce a large amount of hemolysin yet fail to digest meat.

TABLE II.

Comparison of the Proteolytic Activities of Various Species of Streptococci.

Strain No.	Source	Species*	Meat digestion†	Casein digestion‡	Liquefaction of gelatin†	Digestion of blood serum
8616	Erysipelas	*Pyogenes*	+	+	−	−
X 40	Epidemic sore throat	*Pyogenes*§	+	−	−	−
3639	Certified milk	*Pyogenes*	−	+	−	−
8591	Bronchopneumonia	*Pyogenes*	−	−	−	−
3056	Certified milk	*Pyogenes*	+	−	−	−
2735	Certified milk	*Pyogenes*	+	−	−	−
2082	Certified milk	*Infrequens*	+	−	−	−
S 2	Scarlet fever	*Pyogenes*	+	−	−	−
Lexley	Infected ear	*Pyogenes*	+	+	−	−
Lanc.	Impetigo-like lesion	*Equi*	−	−	−	−
8674	Septicemia	*Pyogenes*	+	+	−	−
3636	Certified milk		−	+	−	−
3052	Certified milk	*Infrequens*	+	−	−	−
8556	Postabortion septicemia	*Infrequens*	−	−	−	−
8741	Bronchopneumonia	*Pyogenes*	+	+	−	−
8576	Septicemia	*Pyogenes*	−	−	−	−
Hamer	Sore throat	*Pyogenes*	+	+	−	−

* Holman's classification (12).

† 7 days at 37°C.

‡ 48 hours at 37°C.

§ *S. epidemicus* of Davis (13).

Relation of Tissue Digestion to Liquefaction of Gelatin, Coagulated Blood Serum and Casein.

Eighteen strains of hemolytic streptococci were tested for ability to digest meat, casein, coagulated blood serum and gelatin. To determine gelatin liquefaction, tubes of infusion gelatin were melted in the incubator. These were inoculated with 1 drop of 24 hour bouillon cultures. The coagulated serum was prepared in the form of Loeffler's slants. These were inoculated with 1 drop of the bouillon

cultures above mentioned. The tests for casein digestion were at first made by inoculating tubes of brom-cresol purple milk. Most of these promptly coagulated, and it was difficult to distinguish between small amounts of digestion and separation of the whey. The method was abandoned.

Following the procedure of Eijkman (4), infusion agar was melted in tubes and to each tube, containing about 12 cc. of agar, was added 1 cc. of sterile milk. The mixtures were poured into plates and the surfaces were streaked with the streptococci to be tested. Hydrolysis of the casein is evidenced by a clear area about the colonies which fails to become clouded upon flooding the plate with 25 per cent acetic acid. The results of these tests are recorded in Table II.

From an inspection of this table it is seen that there is no relation between ability to digest tissue and liquefaction of gelatin. This of course applies to the method and strains used. Ability to digest bovine or human tissue does not imply ability to liquefy coagulated bovine serum. Ability to hydrolyze casein is not paralleled by ability to digest tissue, serum or gelatin. The enzyme which digests tissues appears therefore to be distinct from other proteolytic enzymes produced by streptococci.

Nature of the Enzyme.

Ten cultures of hemolytic streptococci in cooked meat medium, all of about the same age (1 month) were selected. Five of these showed considerable digestion. Two were doubtful, the meat level being only about 1 mm. below the line, while the remaining three were distinctly negative. Two were cultures which had shown proteolytic activity only after removal of the vaseline.

Being careful to avoid stirring up the meat, 1 cc. of the clear supernatant fluid was removed from each tube and formol titrations made by Brown's modification (14) of Sörensen's method. This modification is particularly adapted for use with bacteriological media. Table III shows the results of the titrations. It is to be seen in Table III that the cultures showing extensive digestion after 5 days growth at 37°C. also contain large amounts of substances determinable by the formol titration. The extent of the digestion roughly parallels the increase in such substances. The doubtful cultures as well as the

frankly negative ones show little increase in these bodies, the doubtfuls containing little more than the negatives. These results seem to show that the enzyme resembles trypsin in its action.

Further Studies.

To date about 58 strains of hemolytic streptococci have been studied. These represent a variety of sources, bovine, human and otherwise. At present it is sufficient to state that about 30 of them

TABLE III.

Formol Titration of the Supernatant Fluid in Tubes of Cooked Meat Medium Containing Cultures of Various Hemolytic Streptococci.

Strain No.	Approximate per cent of meat digested in 5 days at 37°C.	Quantity of N/20 NaOH needed for formol titration of 1 cc. of the culture
Sterile No. 1	—	0.25
Sterile No. 2	—	0.12
8616	30	4.23
3052*	15	0.55
8741	25	4.85
X 40	25	4.13
8576	5?	0.47
8591	5?	0.41
Lanc.	0	0.40
2082*	12	0.57
4d ign.	0	0.31
S. H.	0	0.40

* Digested meat only after removal of the vaseline seal.

digest meat vigorously. There seems to be no relation between this power and source, pathogenicity or fermentative reactions. A more detailed report of this will be made later.

DISCUSSION.

Proteolysis by streptococci has been observed before. Casease has been frequently described both in culture filtrates and in extracts of washed cells (5, 6). The presence of peptase is well known to be of common occurrence. Prevot (7) has described certain types of

strictly anaerobic streptococci which are capable of breaking down tissue proteins. These organisms produce gas and foul odor and the process appears to be quite unlike that described in this paper. MacCallum and Hastings (8) described a type of proteolytic streptococcus which they named *M. zymogenes*. This organism was obtained from a case of acute endocarditis. It liquefied gelatin, digested coagulated blood serum and peptonized milk. The proteolytic enzymes were found to be active in sterile filtrates. This organism differed from the typical *S. pyogenes* in being practically always arranged in pairs, in its extremely small size and in being able to survive for 3 or 4 months at room temperature upon agar slants in spite of their dried condition. The appearance upon blood agar was not described.

The digestion of tissues, as described in this paper, by ordinary types of hemolytic streptococci seems to be a new observation. The value of such a readily demonstrable characteristic, when exhibited by a considerable percentage of strains lies in its possible use as a means of classification or identification. The tendency in the past has been to rely almost wholly upon the appearance upon blood agar and fermentation reactions. It is only comparatively lately that the use of other cultural characters has become extensive. Among these characters may be mentioned the hydrolysis of sodium hippurate (9) and growth at various surface tensions (10, 11). In view of the unsatisfactory situation existing in connection with identification and classification of the streptococci, it would seem desirable to make more extensive use of such simple tests as hydrolysis of casein, digestion of animal tissues, etc., for these purposes.

SUMMARY AND CONCLUSIONS.

1. An extracellular, proteolytic enzyme has been observed in more than 30 strains of beta type, aerobic and facultative hemolytic streptococci.

2. The enzyme is readily demonstrable in sterile filtrates of cooked meat cultures.

3. No gas or foul odor is produced.

4. It is partially inactivated by exposure to about 60°C. for 45 minutes or longer.

5. The enzyme manifests itself in cooked meat cultures after about 48 hours incubation at 37°C. The sterile filtrate from a 10 day old culture acts within 18 hours.

6. From 50 to 75 per cent of the meat in a tube of cooked meat medium may be digested in about 3 weeks at room temperature after 5 days initial growth at 37°C.

7. No correlation is found, in the cases studied, between hemolysis and proteolysis.

8. Streptococci not digesting beef tissue will not digest human tissue, and those which do digest beef tissue also digest human tissue. This conclusion applies only to the nine strains studied.

9. Ability to digest animal tissues does not necessarily imply ability to digest casein, coagulated beef serum or gelatin.

10. The disappearance of the meat from cooked meat cultures of hemolytic streptococci is quantitatively roughly paralleled by increase of formol-titrable substances in the fluid portion of the medium.

11. The enzyme resembles trypsin in its action. Streptococci from a variety of sources, bovine, human and otherwise have shown varying degrees of proteolytic activity.

12. The name histase is proposed for this enzyme.

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BACTERIUM PNEUMOSINTES IN CLINICAL INFLUENZA IN NEW YORK CITY IN 1926.

By FREDERICK L. GATES, M.D.

In Association with William Owens.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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INTRODUCTION.

Since the first paper in the series by Olitsky and Gates (1) in which the presence of minute filter-passing bodies (*Bacterium pneumosintes*) in cultures from affected rabbits' lungs and from filtered nasopharyngeal washings of influenza patients was reported, a number of investigators in widely separated laboratories have described the isolation, under similar conditions, of anaerobic filter-passing organisms, identified morphologically and sometimes serologically as *Bact. pneumosintes*. Such organisms were "obtained consistently" by Loewe and Zeman (2) in New York, from the filtered nasopharyngeal washings of patients with epidemic influenza, and produced a characteristic clinical and pathological picture (3) when injected into experimental animals. Then Gordon (4) in London reported evidence of the same bacterium in 14 of 20 influenza cases, and filtrates of the bronchial secretion in 3 fatal cases yielded 2 cultures. In 1922 also, Lister (5), in South Africa, obtained 5 cultures of an identical anaerobe in 11 instances in which influenzal washings were cultured within 24 hours of the acute onset, and reported 4 febrile reactions, 1 fever and drop in the leucocyte count, and 1 case of typical influenza among 12 volunteers sprayed with unheated cultures of the filter-passing organism. No reactions occurred among 6 volunteers sprayed with heated cultures. In 1923 Nakajima (6) in Tokio cultivated 2 strains from pharyngeal washings and 1 from the lung tissues of a fatal case of influenza. Seitz (7) in Zurich observed masses of very tiny bodies in the respiratory exudates of influenza patients and grew them for a time in mixed sputum cultures. He regards these bodies as coccoid

rather than bacilloid. Detweiler and Hodge (8) in Toronto grew 3 strains morphologically similar to *Bact. pneumosintes* from filtered influenza material, 2 from lung filtrates of injected animals, 1 from filtered nasopharyngeal washings. Subcultures failed to grow, so identification was not completed. Finally Thomson (9) isolated a minute organism from a case of Engadine fever—a type of influenza endemic in Switzerland—and obtained 2 other strains from influenza patients in England.[1] In all of these studies control experiments with non-influenzal material have been uniformly negative.

Thus the presence of an anaerobic, Gram-negative, filter-passing bacterium, identified as *Bact. pneumosintes*, in the human respiratory tract only in the early hours of an influenzal infection, has been established in many parts of the world. This in itself is but a beginning, however, in determining the relationship of *Bact. pneumosintes* to the clinical disease. A large accumulation of observational and experimental evidence must be sought wherever available, and pieced together as opportunities permit.

The Outbreak of 1926.

When we undertook an investigation of the presence of *Bact. pneumosintes* in clinical influenza in New York City last March (1926), the brief local outbreak proved to be already on the wane. Consequently we had an opportunity to see only a few cases. It was reported that clinically the infections varied considerably in mode of onset, relative prominence of various signs and symptoms, and the blood picture. Yet they would be grouped together with the common designation "influenza" as typified by sudden onset, sometimes with chill, a sharp fever and marked prostration, headache and other pains, absence of profuse coryza, and prolonged depression during convalescence. Sometimes family infections, and more often isolated

[1] While this paper was in press M. W. Hall (*J. Exp. Med.*, 1926, xliv, 539) reported the experimental production of characteristic lung lesions in rabbits and guinea pigs with nasopharyngeal washings from a patient with typical epidemic influenza. From one of his affected animals a culture of *Bact. pneumosintes* was obtained. The presence of this organism in the lungs of experimentally infected animals predisposed them to the pulmonary localization of other bacteria and the production of definite secondary pneumonic lesions.

cases occurred. Compared with the influenza epidemic and its repercussions of 1918 to 1922 this outbreak was characterized in general by the relative mildness of the primary infection and the rarity of secondary complications. Often other signs of typical epidemic influenza, such as flush, photophobia, conjunctivitis, diffuse pharyngitis, and the characteristic leucopenia, were noted, but in the absence of acknowledged proof of the etiologic agent in clinical influenza it would be unwise to draw a close parallel between the infections of this transient outbreak and influenza of the epidemic type. A search for *Bact. pneumosintes* in these sporadic cases thus presented a new field for study and a new problem in its relation to the clinical disease.

In the 10 days that elapsed between our first contact and the failure of available material we obtained nasal and postnasal washings from 9 patients with clinical influenza and from 1 person who developed only a common cold. In several instances the patients had complained of a headache and feeling of depression for a day or two before the acute attack, so that the actual time of invasion is difficult to determine, but the washings were obtained in each instance within 24 hours of the acute onset, marked by fever and prostration in bed. We decided at the outset to follow the established routine in detail and so handled our material as follows:

The patient's nose and throat were washed out with 40 to 50 cc. of sterile, dextrose Ringer's solution. The washings were shaken with beads and divided into two portions. One sample was filtered through a new Berkefeld V candle and used to inoculate Smith-Noguchi medium and *coli* broth (10) under a vaseline seal, and was spread on rabbit blood agar plates for aerobic and anaerobic incubation. The unfiltered nasopharyngeal washings were injected intratracheally by the method of tracheotomy into stock rabbits under light ether anesthesia. The febrile and leucocytic reactions of these rabbits were carefully followed and at autopsy on the 1st or 2nd day, portions of the lung tissue of these animals were ground, and this material, filtered or unfiltered, was inoculated into Smith-Noguchi tubes and on blood agar plates. Fragments were also placed in 50 per cent glycerol, and the rabbits were examined carefully for evidence of concurrent disease.

Rabbit Passages.

Unfiltered washings from 7 of the 9 influenza patients were injected intratracheally into 13 rabbits, usually in amounts of 3 cc. 9 of these

rabbits, representing 6 patients, showed significant reactions, such as fever, a drop in the leucocyte, and especially the monocyte, count, and typical gross and petechial hemorrhages in large, edematous lungs. 6 rabbits injected with whole or filtered lung tissue from 4 of these animals showed similar but less striking effects in the 2nd passage. A 2nd passage apparently failed in 4 other transfers from 2 of the other 5 rabbits.

This series of rabbit injections was carried out under a serious handicap. Our stock of rabbits was low, and the immediate demands of the situation required the use of untested animals. Although only apparently healthy rabbits were chosen for injection, the whole lung tissues of most of them subsequently showed infection with *B. lepisepticus* or *B. bronchisepticus* and cultures of them had to be discarded. In the presence of these concurrent infections a strict interpretation of the reactions of most of these rabbits is not justified. The series was therefore discontinued and our attention turned to the direct cultivation of filter-passing, anaerobic organisms from the nasopharyngeal washings, and from filtered material from the rabbits' lungs.

Cultivation Experiments.

Having in mind that often very sparse growths of filter-passing anaerobes are obtained in early generations, and might easily be missed in the Smith-Noguchi medium, we made at least 2 successive transfers of every primary tube that showed no growth when subplanted on aerobic blood agar plates. The control tubes set up to test the sterility of our media were likewise transferred, to avoid the possibility of false evidence from an extraneous source. Material from each generation was also examined microscopically in stained smears.

This tedious procedure proved to be justified in 6 series of cultures in which minute anaerobic organisms were obtained. 2 of these have been definitely identified as strains of *Bact. pneumosintes*. In the first 2 generations of these *pneumosintes* cultures the growth was so sparse as to escape microscopic detection and no visible colonies developed in subplants on anaerobic blood agar plates. But in the 3rd, 4th, and subsequent generations the typical clouding of the Smith-

Noguchi medium, the microscopic observation of minute, Gram-negative bodies such as have been fully described (10), and the growth on anaerobic blood agar plates of microscopic, discrete, round, convex colonies with an entire edge and a colorless translucency indicated the growth of *Bact. pneumosintes* morphologically identical with the 1918 to 1922 strains.

One strain was obtained from the whole lung tissue of a rabbit injected with unfiltered nasopharyngeal washings, and consequently had not been filtered. Fortunately this rabbit was free from previous lung infection and, as in numerous cases reported by Olitsky and Gates (11), the contaminating bacteria (in this case *S. albus* and diphtheroids) in the unfiltered nasopharyngeal washings were suppressed during the rabbit passage.

The 2nd strain, also obtained through rabbit passages, was derived from the filtered lung tissue of a 2nd passage rabbit, intratracheally injected with whole lung tissue that had stood in 50 per cent glycerol for 31 days. This 1st passage lung tissue had been contaminated with a large Gram-negative bacillus which did not survive glycerolation, so that the lungs of the 2nd rabbit yielded no aerobic growth. The 2nd passage rabbit showed no fever, only a slight leucopenia (a drop in monocytes from 2790 to 2040 cells), and no gross lesions except one small surface hemorrhage in the lungs.

No primary cultures of *Bact. pneumosintes* were obtained in *coli* broth or on anaerobic blood agar plates. These media are only suitable for special purposes with well established strains. This fact emphasizes the importance of the Smith-Noguchi medium for primary cultures, and even in this medium the initial cultivation of *Bact. pneumosintes* is difficult and uncertain. A lesson may be drawn from the detection of this fastidious organism only in the 3rd generation of culture and even then only after 1 or 2 preliminary rabbit passages. It was our earlier experience that the cultivation of *Bact. pneumosintes* was more frequently successful from the lung tissues of affected rabbits than directly from the filtered nasopharyngeal washings of influenza patients.

2 other strains of anaerobic, filter-passing organisms morphologically similar to *Bact. pneumosintes* were obtained in Smith-Noguchi medium directly from the filtered nasopharyngeal washings of other

influenza patients. The 1st generations grew so sparsely as to escape detection and the bacteria were first discovered as submicroscopic colonies in subplants on anaerobic blood agar plates. Although these 2 strains have grown well in successive generations on solid media in the anaerobic jar, they have both died out in the Smith-Noguchi tubes and repeated attempts to reestablish growth in fluid media have so far failed. The morphological similarity to *Bact. pneumosintes*, the very minute, discrete colonies on blood agar plates, and the failure to develop in successive transplants on fluid media are characteristic of the Group II organism briefly described by Olitsky and Gates in 1922 (12). These strains have not yet been grown in sufficient quantity for serological examination.

In addition to these 4 morphologically similar organisms, which may all belong to a common group, 2 other anaerobic filter passers were isolated directly from washings of influenza patients. As in the earlier studies, the identification of these 2 other organisms depended on the use of anaerobic blood agar plates on which they grow readily in visible colony form. The primary cultures were obtained in Smith-Noguchi medium; in one instance also in *coli* broth. One strain is apparently a variant of Group I, the other is similar to the organisms described as Group III (12).

Serological Reactions.

At the beginning of this investigation 6 rabbits were set aside for immunization with old strains of *Bact. pneumosintes* (C 17 and C 34) from 1919 and 1922. The organisms were grown in *coli* broth, washed, standardized, and injected subcutaneously in large doses at weekly intervals until 7 or 8 injections had been given. The rabbits then yielded serum with a complete agglutination titer of 1:160 to 1:320 against the old strains. These titers are the highest that have yet been obtained with these organisms and may indicate an increase both in agglutinogenic properties and in response to serum antibodies on prolonged saprophytic cultivation.

The 2 new strains of *Bact. pneumosintes* show a strictly specific agglutination in low dilutions, 1:2 to 1:20, of this anti-*pneumosintes* serum.

At intervals of 15 to 30 days after the acute onset, serum samples were obtained from 8 of the 9 influenza patients from whom washings had been taken, and from 9 other convalescents from clinical influenza. As controls 10 samples were taken from normal persons who said that within a year they had not had any acute respiratory infec-

TABLE I.

Agglutination Tests with Convalescent and Normal Human Sera.

Convalescent sera					Normal sera				
	Bact. pneumosintes strain					*Bact. pneumosintes* strain			
	17	34	49	50		17	34	49	50
Serum dilutions	1:10	1:10	1:2	1:2		1:10	1:10	1:2	1:2
1	+++	++	++	++	1	+++	++	−	
2[a]	−	−	−		2	+	−	−	−
3[b]	++	+	++		3	+++	−	++	++
4	++	+++	+	+	4	+++	+	++	+
5[c]	+++	++	++		5	−	−	−	−
6[d]	++	++	++	+	6	+++	++	−	
7	++	++			7	−	−		
8[d]	+++	++	+	+	8	−	−		
9	+++	++	+		9	+++	−	−	−
10	+++	+	++	++	10	−	−		
11	+++	+	−	+	Normal rabbit	−	−	−	−
12	+++	++	−						
13	+++	+	+	++					
14	+++	++	−						
15	++	++	++						
16	++	+	+						
17	++	−							

[a] Group III organism recovered.
[b] *Bact. pneumosintes* recovered.
[c] Group I organism recovered.
[d] Group II organism recovered.

tion diagnosed as influenza. These sera were tested for specific agglutinins, by the method previously described (13), against 2 old strains (Nos. 17 and 34), and against the 2 new strains of *Bact. pneumosintes* (Nos. 49 and 50), so far as the very limited amounts of available material permitted.

A summary of these agglutination tests (Table I) shows that the serum of only 1 patient with a clinical diagnosis of influenza failed to

agglutinate 1 or both old strains of *Bact. pneumosintes*, and when tested, most of them (12 of 15) agglutinated 1 or both of the 1926 strains also. Considered together with the agglutination of the new strains by specific anti-*pneumosintes* rabbit serum, this evidence points to an immunological relationship as well as a morphological identity between the 1919 to 1922 and the 1926 strains. Heretofore agglutination of *Bact. pneumosintes* even after a long saprophytic existence in the laboratory has not been found in the serum of supposedly normal persons. In these tests unquestioned agglutination of old and new strains sometimes occurred. Several explanations of this phenomenon are possible, but we shall not attempt to develop any of them at this time, on the basis of the evidence available at present.

SUMMARY.

The presence of *Bacterium pneumosintes* has been demonstrated in nasopharyngeal washings from 2 patients in a sporadic outbreak of clinical influenza in New York City in March, 1926. 2 strains of bacteria morphologically similar to *Bact. pneumosintes*, but differing in certain cultural characters, and 2 other anaerobic filter-passing organisms were also isolated from the 9 patients examined.

The blood serum of 16 among 17 persons convalescent from clinical influenza, and of 6 among 10 supposedly normal persons, agglutinated 1 or more strains of *Bact. pneumosintes*.

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EXPERIMENTAL HIGH JEJUNOSTOMY IN THE DOG, WITH BLOOD CHEMICAL STUDIES.

By RUSSELL L. HADEN, M.D., and THOMAS G. ORR, M.D.

(*From the University of Kansas School of Medicine, Kansas City, Kansas.*)

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In a recent publication (1), we have discussed the effect of jejunostomy upon experimental obstruction of the jejunum. This work was stimulated by the interest manifested in jejunostomy in recent years as a treatment of clinical intestinal obstruction and peritonitis. The following experiments have been made to study the effect of of simple high jejunostomy upon the life of the dog and the chemistry of its blood. A comparison is made between the results of high jejunostomy and low ileostomy.

Method.

All operations were done under ether anesthesia. The jejunostomy was made about 12 inches below the ligament of Treitz. A modified Witzel technique was used in some cases and in others the gut was sutured to the peritoneum and skin and opened. Low ileostomies were done with the same technique. Drainage was prompt and continuous in all cases. Blood for analysis was withdrawn from the jugular vein. All animals showing complications at autopsy were rejected. The non-protein nitrogen was determined by the method of Folin and Wu (2), the urea nitrogen by the Van Slyke and Cullen (3) modification of the Marshall method, and the carbon dioxide-combining power by the method of Van Slyke and Cullen (4). The chlorides were determined on the tungstic acid filtrate in the manner suggested by Gettler (5).

EXPERIMENTAL OBSERVATIONS.

The effects of simple jejunostomy were first studied. Six animals lived from 2 to 5 days following the jejunal drainage. In every instance the blood showed a rapid rise in the non-protein and urea nitrogen. These changes are similar to those observed in simple obstruction of the jejunum (1) but develop more rapidly. In four

animals the blood chlorides showed a decrease. The carbon dioxide-combining power did not show any constant change (Table I).

TABLE I.

Simple Jejunostomy 12 Inches below Ligament of Treitz.

Dog No.	Day after operation	Blood				Treatment
		Amount per 100 cc.			CO_2-combining power	
		Total non-protein nitrogen	Urea nitrogen	Chlorides		
		mg.	*mg.*	*mg.*	*vol. per cent*	
1	0	34.6	9.8	510	41.9	Operation
	1	37.5	10.5	460	46.6	
	2	31.2	13.0	450	40.9	
	3	49.2	18.2	460	34.3	
	4	134.0	72.7	450	33.4	Died
2	0	27.3	8.4	470	36.2	Operation
	1	30.0	14.0	490	28.5	
	2	52.8	26.6	470	24.7	Died
3	0	24.6	10.5	490	36.2	Operation
	1	40.0	20.3	430	38.0	
	2	129.0	66.5	370	29.4	
	3	218.0	115.5	320	29.4	Died
4	0	26.5	8.4	490	24.7	Operation
	1	31.6	8.4	440	29.6	
	2	67.0	22.6	360	22.8	
	3	68.3	27.9	340	29.4	
	4	92.5	46.2	340	30.5	
	5	159.0	81.2	340	25.6	Died
5	0	31.6	10.5	520	20.9	Operation
	1	37.5	9.1	430	28.5	
	2	115.0	43.4	430	16.2	
	3	228.0	101.5	430	21.9	Died
6	0	30.6	14.7	470	40.0	Operation
	1	45.0	22.4	410	40.0	
	2	79.8	41.3	390	43.8	Died

In two control dogs jejunostomy was done as indicated above and the drainage tube completely closed to prevent drainage at any time.

TABLE II.

Jejunostomy—Tube Obstructed and No Drainage Permitted.

Dog No.	Day after operation	Blood				Treatment
		Amount per 100 cc.			CO_2-combining power	
		Total non-protein nitrogen	Urea nitrogen	Chlorides		
		mg.	*mg.*	*mg.*	*vol. per cent*	
7	0	30.0	8.4	410	36.2	Operation
	1	33.0	11.2	440	36.2	
	2	30.6	9.8	450	31.5	
	3	34.5	8.4	460	36.2	
	4	29.7	9.8	460	29.4	
	5	70.5	27.3	420	27.5	
	6	32.3	23.8	450	29.4	
	7	37.0	10.5	470	32.4	
	8	24.6	11.8	470	35.3	
	9		7.0	470	29.6	
	10	26.8	11.2	490	34.3	
	12	24.8	9.1	530	32.5	Feeding started
	15	30.9	14.0	530	33.4	
	17	36.6	11.9	510	28.5	
	19	28.2	21.6	520	25.6	
	23	25.7	5.6	520	32.4	Drainage tube removed
	26	33.0	19.6	510	34.3	Dog well
8	0	25.2	11.8	490	34.3	Operation
	1	31.6	11.8	460	31.5	
	2	30.0	7.0	370	24.7	
	3	30.0	12.6	460	30.5	
	4	23.6	7.7	470	36.2	
	5	27.3	13.3	490	27.5	
	6	27.5	20.3	480	31.5	
	7	30.3	5.6	500	31.5	
	8	21.6	7.0	520	32.4	
	9	30.0	9.1	520	29.4	
	10	29.7	11.2	530	32.4	
	11	24.6	10.5	540	31.5	Tube removed
	12	22.4	9.1	560	31.5	
	13	22.9	8.4	570	31.5	
	15	30.0	6.3	540	34.3	Feeding started
	17	28.5	8.4	600	30.5	
	19	23.0	6.3	550	29.4	
	23		7.0	560	33.4	Dog well

TABLE III.

Ileostomy 12 Inches above Cecum.

Dog No.	Day after operation	Blood				Treatment
		Amount per 100 cc.			CO_2-combining power	
		Total non-protein nitrogen	Urea nitrogen	Chlorides		
		mg.	*mg.*	*mg.*	*vol. per cent*	
9	0	23.0	6.3	430	39.0	Operation. Rubber tube drainage
	1	34.5	6.3	450	42.8	
	2	33.3	9.1	460	39.0	
	3	31.6	11.2	460	40.0	
	4	24.4	11.2	460	33.4	
	5	26.8	11.9	440	35.3	
	6	23.6	7.7	480	35.3	
	7	30.9	16.8	480	30.5	
	8	37.0	11.9	530	37.2	
	9	34.5	17.5	450	32.4	
	10	41.6	11.9	520	33.4	
	11	29.1	7.7	480	29.4	
	12	29.4	9.1	540	36.2	
	13	23.3	7.0	530	34.3	
	14	23.4	6.2	580	26.6	Tube removed
	16	27.5	7.0	570	30.5	Dog recovered
10	0	33.0	7.0	460	29.6	Operation. Rubber tube drainage
	1	50.0	11.9	450	29.4	
	2	33.3	7.0	480	29.4	
	3	31.9	17.5	440	29.6	
	4	33.0	11.2	480	39.0	
	5	33.0	16.8	460	32.4	Dog pulled tube out. Ileum sutured to skin and opened
	6	27.3	10.5	460	36.2	
	7	27.0	8.4	440	38.1	
	8	30.9	11.2	480	31.5	
	9	31.6	8.4	480	37.2	
	10	23.0	5.6	470	32.4	
	11	31.6	2.1	450	34.3	
	12	29.1	11.2	530	34.3	Began feeding bread
	13	33.3	11.3	490	36.2	
	14	33.3	9.1	480	30.5	
	15	22.9	6.1	500	27.5	
	20	31.2	11.2	550	27.5	
	25	33.3	19.6	560	32.4	
	28	25.2	11.2	600	36.2	
	34	31.9	13.3	560	35.3	No further blood studies. Dog died in 55 days

TABLE IV.

Simple Jejunostomy. Treatment with Sodium Chloride Solution.

Dog No.	Day after operation	Blood: Amount per 100 cc. Total non-protein nitrogen	Urea nitrogen	Chlorides	Blood: CO_2-combining power	Treatment
		mg.	*mg.*	*mg.*	*vol. per cent*	
11	0	26.3	5.6	490	34.3	Operation
	1	42.8	10.2	430	34.3	40 cc. per kg. of 0.8 % NaCl subcutaneously
	2	42.8	9.1	460	37.2	" " " " " " " " "
	3	31.6	9.1	450	29.4	" " " " " " " " "
	4	33.3	9.1	500	31.5	" " " " " " " " "
	5	33.3	9.1	560	25.6	" " " " " " " " "
	6	29.7	6.4	580	29.6	" " " " " " " " "
	7	56.4	24.5	630	30.5	Died
12	0	31.6	7.0	440	27.5	Operation
	1	26.3	8.4	450	27.5	40 cc. per kg. of 0.8% NaCl subcutaneously
	2	30.6	9.8	500	25.6	" " " " " " " " "
	3		21.7	540	23.8	" " " " " " " " "
	4	25.0	13.3	560	25.6	" " " " " " " " "
	5	25.0	11.9	470	25.6	" " " " " " " " "
	6	36.1	14.7	600	22.8	" " " " " " " " "
	7	34.1	18.9	690	20.9	Died
13	0	28.2		510	40.0	Operation
	1	29.1	10.5	400	36.2	950 cc. of 0.8% NaCl subcutaneously
	2	32.3	5.6	550	34.3	
	3	25.0	9.8	490	31.5	1920 " " " " " "
	4	32.3	14.8	560	10.7	
	5	30.9	19.6	600	20.0	
	6	60.0	11.2	700	21.9	700 " " " " " "
	7	114.0	49.8	750	16.2	Died
14	0	34.9	9.1	430	31.5	Operation
	1	33.3	10.5	430	32.4	800 cc. of 0.8% NaCl subcutaneously
	2	33.0	5.6	510	30.5	
	3	31.2	7.9	490	29.4	600 " " " " " "
	4	26.1	11.9	530	30.5	
	5	28.0	9.1	520	25.6	
	6	41.6	22.9	640	18.1	500 " " " " " "

TABLE IV—*Concluded.*

Dog No.	Day after operation	Blood				Treatment
		Amount per 100 cc.			CO_2-combining power	
		Total non-protein nitrogen	Urea nitrogen	Chlorides		
		mg.	*mg.*	*mg.*	*vol. per cent*	
14	7	39.5	11.2	550	28.7	
	8	28.5	16.1	500	32.4	
	9	36.1	14.0	560	28.7	500 cc. of 0.8% NaCl subcutaneously
	10	68.0	30.8	580	28.7	
	11	42.8	22.4	530	34.3	400 " " " " " "
	12	43.6	23.8	640	32.4	" " " " " " "
	13	71.3	26.6	700	30.5	Died

These animals lived indefinitely without the development of the chemical changes in the blood noted in simple jejunostomy. In 10 days to 2 weeks the tubes loosened and were removed or dropped out and the wounds healed. These findings indicate that the operative procedure alone would not cause death or marked changes in the chemistry of the blood (Table II).

Ileostomy 12 inches above the cecum was done in four dogs. Three of these lived 16, 29 and 55 days and the fourth was still alive after 72 days when studies were discontinued. No constant changes in the blood chemistry were noted (Table III).

Four dogs with simple high jejunostomy were treated with sodium chloride solution. They lived from 7 to 13 days. Two were treated by giving physiologic sodium chloride solution in daily doses of 40 cc. per kilo of body weight. An effort was made in the other two to replace with sodium chloride solution the weight lost from day to day. In every instance the sodium chloride of the blood was above normal at the time of death. A definite rise in non-protein and urea nitrogen of the blood also occurred before death (Table IV).

Water by mouth was not given in these treated dogs. In spite of the quantity of liquid given subcutaneously a marked and rapid loss of weight occurred in each animal.

DISCUSSION.

Dogs with simple high drainage of the jejunum lived from 2 to 5 days. This is a shorter average length of life than with simple obstruction at the same location. The chemical changes of the blood are similar to those occurring in high intestinal obstruction (6). They differ however from pyloric (7) and duodenal obstruction in that the carbon dioxide-combining power does not show any constant change and the chlorides do not show such a marked fall.

Animals with simple jejunostomy and closure of tube to prevent drainage proved that the operation done was not the cause of death in these cases.

Ileostomy 12 inches above the cecum did not produce the profound disturbance of high jejunostomy. It is probable that some dogs would live indefinitely with low drainage of the ileum.

Treatment with sodium chloride solution definitely prolongs the life of dogs with high jejunostomy. The cause of the rapid death in high jejunostomy apparently differs from that due to high intestinal obstruction. Sodium chloride solution will not protect in this condition to as great an extent as in high intestinal obstruction (8).

Whether cause of death is due to dehydration, toxemia, loss of chlorides or loss of other important elements is not known. Fasting is not to be considered because of the short duration of life.

CONCLUSIONS.

1. The effect of high jejunostomy upon the life and chemical changes of the blood of dogs is here reported.

2. Sodium chloride solution administered in sufficient quantity after high jejunostomy prolongs the life of dogs.

3. Such experimental findings as these warrant a careful clinical study of the effects of high jejunostomy so frequently used in the treatment of acute intestinal obstruction.

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STUDIES ON THE ETIOLOGY OF HEARTWATER.

III. The Multiplication of Rickettsia ruminantium within the Endothelial Cells of Infected Animals and Their Discharge into the Circulation.*

By E. V. COWDRY.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

Plate 31.

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The first two of these studies[1] dealt with the discovery of a new species of *Rickettsia* in the tissues of sheep, goats and cattle suffering from heartwater and in the ticks which transmit it. In this third paper, it is proposed to describe in detail the behavior of the *Rickettsiæ* after they have entered the endothelial cells of the blood vessels of infected animals.

The chief obstacle to the acceptance of *Rickettsiæ* as the inciting agents of disease in man is the difficulty of demonstrating their existence clearly and regularly in the lesions. This is almost insurmountable in the case of trench fever, because it is seldom if ever, fatal, so that material for examination cannot be collected post mortem, unless death should accidentally occur at a propitious moment from some other cause, which in practice has not happened. In two other arthropod-transmitted diseases—typhus and Rocky Mountain spotted fever—*Rickettsiæ* are undoubtedly present in the tissues, but only in very small numbers. Their distinctive properties are obscured by extensive vascular degenerations resulting in the formation of many granules which, like them, are colored feebly by basic dyes. Since, moreover, all three species of *Rickettsiæ* are smaller than most

* Sixth contribution by the South African Expedition of The Rockefeller Institute for Medical Research.

The experiments were made and material collected at the Government Laboratories at Onderstepoort and cordial thanks are due to the Government of the Union of South Africa, to Sir Arnold Theiler and to the members of his staff for the many courtesies extended. The study herein reported was made after my return to the United States.

[1] Cowdry, E. V., *J. Exp. Med.*, 1925, xlii, 231, 253.

bacteria, and stain much less intensely, it is not surprising that so little is known of their exact relation to the tissues of man and experimentally infected animals.

It is believed that heartwater affords an unusual opportunity for securing information on this particular aspect of the general problem of *Rickettsiæ* and of their relation to disease.[2] As already noted degenerative vascular lesions are almost entirely absent in heartwater, and the *Rickettsiæ* themselves, while very sparsely distributed in the tissues as compared with some bacteria in other conditions, are nevertheless much more abundant than the *Rickettsiæ* in any of the above mentioned infections. Another advantage offered by the heartwater *Rickettsiæ* is that they may be colored, without great difficulty, by a variety of stains in addition to that of Giemsa on which chief reliance must be placed for the study of the other *Rickettsiæ*.

Despite the unusual facility with which the heartwater *Rickettsiæ* may be observed and studied, they resemble other *Rickettsiæ* closely in many important particulars. Like them they are very small, Gram-negative organisms (resisting attempts at cultivation on artificial media) which are found principally in the endothelial cells of the blood vessels of diseased animals. All are transmitted by the bites of insects or arachnids, and the conditions to which they give rise are similar in so far as they are acutely febrile, the nervous system is frequently involved and they confer an immunity lasting for several years or for life. The chief point in which heartwater differs from typhus fever and Rocky Mountain spotted fever is in the absence of cutaneous lesions, which is probably correlated with the circumstance already mentioned, that there is but slight vascular involvement.

EXPERIMENTS.

Most of the animals studied were infected by the intrajugular inoculation of blood from cases of heartwater; but some were infected by allowing ticks, carrying the virus, to feed upon them. They were sacrificed at appropriate intervals during the incubation period, and febrile stages, and during convalescence (in the rare cases which did not terminate fatally). A detailed account of the experiments, together with the autopsy reports, has already been given in the first two papers.

Rickettsiæ in the Circulating Blood Stream.

That the virus, and presumably therefore the *Rickettsiæ*, are present in the blood stream during the febrile stages and for about 6 days after the temperature has commenced to decline in animals which have not

[2] Cowdry, E. V., *Arch. Path. and Lab. Med.*, 1926, ii, 59.

succumbed, was shown by the inoculation of blood into other susceptible animals. But although many attempts were made, it was not possible to discover the *Rickettsiæ* in blood smears. This failure corresponds with the experience of investigators while studying the blood of cases of trench fever and of typhus fever. A few heartwater *Rickettsiæ* were, however, detected within the vascular lumina of tissues examined in sections. The most probable explanation is that they do not occur in the blood stream in large masses but as single individuals, or perhaps in the form of very small clumps (Fig. 7). Obviously even the most careful differentiation of the stain might completely bleach single elements of such small dimensions (only about 0.25 μ in diameter), whereas the large aggregates within the endothelial cells would probably retain the dye more tenaciously, owing to its reduced rate of diffusion.

Penetration of the Rickettsiæ into the Endothelial Cells.

The actual passage of *Rickettsiæ* through the cell membrane was never seen, but there is reason to believe that several may enter a single cell, most likely in sequence.

The sites of entry and the foci of subsequent multiplication are variable. They may penetrate the cytoplasm at a point where it consists of a thin film almost invisible microscopically, relatively remote from the nucleus. Or, they may gain admittance to the cytoplasm lying between the endothelial nucleus and the lumen of the blood vessel (Fig. 2). In rare cases they may migrate through the cytoplasm to a point on the opposite side of the nucleus, that is to say between it and the periphery, remote from the lumen (Fig. 5).

Multiplication in such a location, proximal to the endothelial nuclei, has not thus far been reported in the case of any of the other pathogenic *Rickettsiæ*. Yet it might be expected to occur, because the *Rickettsiæ* of both typhus and Rocky Mountain spotted fever possess much greater power of penetration than the heartwater *Rickettsiæ*. They even enter the successive vascular tunics and frequently gain an extravascular position, whereas those of heartwater are apparently definitely and invariably confined to the vascular lumen and its lining endothelium.

Before considering the probability of sequence in penetration, reference must be made to an observation which was briefly mentioned in the first paper, namely, that many spherical aggregates of *Rickettsiæ* may develop within a single endothelial cell. Their number may reach as many as eight. In Fig. 1 six are illustrated within the limits of a section only 3 microns in thickness. The colonies invariably remain distinct and never fuse, even when closely applied to each other.

The clumps inhabiting one cell are seldom of the same size. Their dimensions may vary from about 0.5 μ to 25.0 μ as observed after fixation and staining, and the number of *Rickettsiæ* from two to several hundreds. Sometimes the *Rickettsiæ* in the smaller ones are slightly larger and color more deeply than those grouped in the larger masses (Figs. 1 and 6). Although such a distinction is by no means of constant occurrence, it does seem probable that the smaller colonies are younger than the larger ones,—that they had less time to multiply—in other words, that there has, in fact, been a sequence in the entry of *Rickettsiæ*.

Just why the *Rickettsiæ* should enter some endothelial cells in marked preference to others is not known. It does not seem to take place *pari passu* with the phagocytosis of broken down erythrocytes, for example. In heartwater there is very little taking up of erythrocytic debris by the endothelial cells of the cerebral cortex, and kidney, which nevertheless harbor *Rickettsiæ* more constantly than the cells of any other tissues. In the rare cases when it is phagocytized in these localities the cells which do so have never been observed to accumulate *Rickettsiæ* as well, but it would be unsafe to assert that such a double acquisition is impossible. It is conceivable that there may be an element of antagonism in the two processes. Thus, the *Rickettsiæ* were never seen in the endothelial cells of the liver, and they are of very inconstant occurrence in those of the spleen, both of which are noted for the avidity with which they phagocytize erythrocytes and materials of foreign origin generally. Neither is the liver the best place to search for the *Rickettsiæ* of typhus and Rocky Mountain spotted fever, although they do occur in it sparingly. Experimental blocking of the endothelium in heartwater with particulate matter might perhaps modify its reaction to the *Rickettsiæ*.

The *Rickettsiæ* of heartwater resemble those of typhus fever in this predilection which they have for the brain, while they differ in other minor points of distribution, such as their failure to accumulate in very large numbers in the skin, or the testicle, and the peculiar attraction which the kidney seems to exercise over them.

Growth of the Rickettsiæ within the Endothelial Cells.

A single isolated *Rickettsia* has never been seen within an endothelial cell; but in Fig. 5 a *Rickettsia* in the act of division, or two *Rickettsiæ* in especially close contact, are illustrated. In very thin sections (1 μ) it is possible to distinguish similar formations suggestive of simple transverse fission, and it is probable that this process takes place commonly and at a very rapid rate. Sometimes the *Rickettsiæ* are congregated at the periphery of the colony in such a manner as to suggest the interpretation that they divide in a plane perpendicular to its surface and that the growth is peripheral rather than central (Fig. 3).

Some striking examples of growth pressure were found. Fig. 2 illustrates a colony which has developed between the nucleus and the vascular lumen. While growing it has pressed upon the nucleus, forming a marked concavity in it. A rarer instance, which, indeed, has only once been observed so clearly, is still more significant (Fig. 4). It consists of two colonies of approximately the same size (and age) which have developed at opposite poles of the nucleus of an endothelial cell. When the *Rickettsiæ* first commenced to multiply, this nucleus was presumably flattened by arterial blood pressure in a direction parallel to the length of the vessel, like its neighbors. The pressure which the *Rickettsiæ* have exerted has completely altered its position so that it has been forced to stand on its head, so to speak. This can only mean that the growth pressure of the *Rickettsiæ* actually exceeds the arterial blood pressure because it is sufficient to cause a nucleus, which is ordinarily flattened by the blood pressure, to expand directly against it. To the best of my knowledge no alteration like this is produced through the phagocytosis of lifeless materials.

The observation that when two colonies grow toward each other and come in contact the area of separation between them is a plane

surface indicates that their growth pressure is about equal. If it were greater in one than in the other, only one would become flattened (*i.e.*, that in which the pressure was least), or else the one endowed with the largest expanding force would indent the other. It is possible, thus, to ascertain that the growth pressure of small colonies is approximately the same as that of larger ones.

Throughout this period of multiplication the morphology and staining reactions of the *Rickettsiæ* vary only within the limits already alluded to. Figs. 3, 4 and 6 illustrate their appearance by Giemsa's method; Figs. 2 and 5 by hematoxylin and eosin; Fig. 7 by Loeffler's methylene blue; and Fig. 1 after staining with eosin and methylene blue. The variability in color of some of the *Rickettsiæ* is probably caused by fortuitous differences in the rate of extraction of the stains, some of the *Rickettsiæ* being nearer than the others to dye-holding materials or to the surface of the section.

Thus, in their tinctorial properties also, they resemble the *Rickettsiæ* of typhus and Rocky Mountain spotted fever, though they are more easily stained. In all three the differentiation into red- and blue-staining materials, which may be observed in smears of the arthropod vectors, is not distinguishable in sections of infected mammalian tissues.

That the *Rickettsiæ* of heartwater are organisms possessed of definite internal organization, is shown by the fact that they retain their spherical shape and do not tend to flatten out when they come in touch with the cell wall and other cellular components. Yet with a very high magnification (of 4000 diameters or more) their outlines become more hazy than do those of the mitochondria, for instance. This would lead one to suppose that, if they possess an organized limiting membrane, it is of somewhat rudimentary character as compared with that of typical bacteria.

Only in an animal's tissues which have not been promptly excised after death, are any signs of the degeneration of *Rickettsiæ* to be noted. When, under such conditions, degeneration sets in the *Rickettsiæ* begin to exhibit considerable variability in size and to lose their peculiar tinctorial properties. The forces which have previously kept them spacially arranged in respect to their fellows cease to operate and they tend to agglutinate in rather amorphous masses. Though

unquestionably dead, they may still be recognized for 6 hours or more after the death of the animal containing them, chiefly by their topographic relationship to the blood vessels.

Reaction of the Endothelial Cells to the Presence of the Rickettsiæ.

Unless the living endothelial cells contain *Rickettsiæ*, they show no signs of injury. In this respect the endothelial reaction in heartwater differs sharply from that which may be observed in typhus and Rocky Mountain spotted fever. In these two diseases the endothelial cells in which the *Rickettsiæ* are absent become altered almost as regularly and to about the same degree as those which harbor many of them.

When the *Rickettsiæ* of heartwater do enter the endothelial cells and multiply within them, they do not cause very serious injury. The principal alteration which the cells undergo is great mechanical distention to accommodate the *Rickettsiæ* in large numbers. Their nuclei seldom, if ever, show definite signs of injury. Karyokinetic figures are of very rare occurrence and there is no evidence of amitosis. It is possible that the mitochondria and Golgi apparatus in the cytoplasm are altered; but they cannot be greatly damaged, because the cells continue to live. No signs whatever of basophilic degeneration, like that which occurs in typhus and Rocky Mountain spotted fever, were seen. Indeed, the cytoplasm, although swollen and greatly increased in extent, reacts in the usual way to all ordinary stains except for certain foci, in which the *Rickettsiæ* are multiplying, which become chromophobic.

These vacuole-like spaces containing *Rickettsiæ* are filled with a clear watery fluid, probably saline in nature, as the action of a wide range of fixatives fails to reveal any coagulable substance in it. The margins of these spaces are sharply defined and do not grade into the surrounding cytoplasm. Morphologically the spaces do not differ from areas of intracellular digestion containing broken down red blood cells, but biochemically they are probably dissimilar, for the reason that there can be but little in common between the process of intracellular digestion and the conditions which facilitate the multiplication of *Rickettsia*-like organisms.

The *Rickettsiæ* of heartwater are usually observed clumped in the central parts of these spaces. They do not become applied to

the interface between the fluid contents of the spaces and the cytoplasmic environment. It is interesting to note that neighboring spaces communicate with one another, as illustrated in Figs. 1 and 6; also, that a single space may house more than one colony of *Rickettsiæ* (Fig. 1).

The reason why these colonies of *Rickettsiæ*, suspended in a fluid medium within a single vacuole-like container, or communicating system of containers, of microscopic dimensions, should retain their individuality instead of coalescing to form a larger mass, is not easily discovered. One would expect them to be thoroughly mixed by the continual changes in size of the pulsating vascular walls, but they are equally discrete in veins, which do not pulsate, as in arteries.

In sectioned mammalian material, it has not been possible thus far to bring to light any indications of the existence of a matrix, or binding material, which might restrict or retard the movement of the individual organisms forming a colony. But, in the second paper, on the *Rickettsiæ* of heartwater as they appear in infected ticks,[3] some clumps of *Rickettsiæ* were found to be embedded in such a ground substance which differed slightly in staining properties from the cytoplasm of the host cells. This was best seen after formalin fixation. When, moreover, portions of the tick's alimentary tract were teased out in an approximately isotonic medium the individual *Rickettsiæ* in a clump were found to cohere together despite considerable mechanical traction. Two possibilities therefore present themselves; either a matrix of this kind is absent in the tissues of infected mammals, or else it is present in such a form that it eludes our methods of demonstration. It may be an inconstant accompaniment of intracellular *Rickettsial* growth, and exist in very small amounts in the case of *Rickettsia prowazeki* in which the "globular massing" is so much less distinct.

Discharge of Rickettsiæ from the Endothelial Cells into the Blood Stream.

After the incubation period has passed and the febrile reaction is at its height, the endothelial cells often rupture and discharge their contents into the circulation (Figs. 4 and 6). Sometimes the broken

[3] Cowdry, E. V., *J. Exp. Med.*, 1925, xlii, 253.

edges of the cell membrane may be distinguished so that it is certainly not a case of the entry of *Rickettsiæ*. That we are not dealing with a dragging out of the *Rickettsiæ* by the microtome knife (as occasionally happens in the case of resistant bodies, like nucleoli) is indicated by the absence of scratches and by the observation that in one and the same section the discharge may take place in several directions. As far as our microscopic evidence goes, these liberated *Rickettsiæ* have every appearance of being just as viable as those which originally entered the cell. No parallel instance of the discharge of *Rickettsiæ* into the blood stream has been reported in Rocky Mountain spotted fever or in typhus fever. A special search which I have made myself of tissues very kindly given to me by Drs. Wolbach, Rajchmann and Nicholson has failed to reveal any indications of a similar process.

Even with localized cellular injury of this extent and degree, the nucleus remains apparently normal and the cell continues to live. This normality of the endothelium constitutes another point in favor of the conclusion that the *Rickettsiæ* live and actively multiply within the vascular endothelial cells. By contrast, the extensive endothelial injury in typhus and Rocky Mountain spotted fever seems to be correlated, at least in part, with the death of many *Rickettsiæ* within the cells and the resultant liberation of substances which may be poisonous. In other words, the endothelial inclusion of *Rickettsiæ* is not so much a mechanism of defense in heartwater as it appears to be in these other diseases.

DISCUSSION.

The *Rickettsiæ* of heartwater exhibit certain features typical of other pathogenic *Rickettsiæ* with almost exaggerated clearness.

The most characteristic appearance of *Rickettsiæ* in human lesions is, according to Wolbach, Todd and Palfrey,[4] their tendency to globular massing. In heartwater this property of forming spherical clumps within the endothelial cells is even more conspicuous. These spherical masses, or colonies, grow within vacuole-like cytoplasmic spaces filled with a clear fluid. Further data are needed concerning these spaces, because clues might thereby be secured for the preparation

[4] Wolbach, S. B., Todd, J. L., and Palfrey, F. W., The etiology and pathology of typhus, Cambridge, 1922.

of artificial media suitable for the cultivation *in vitro* of the heartwater *Rickettsiæ* and conceivably for those of typhus and Rocky Mountain spotted fever also. Many difficulties would be encountered in making such an analysis, but indicators might be of service, especially the *in vivo* synthesis of Prussian blue. The oxydase reaction should be applied. The fact that multiplication appears to attain a maximum during the height of the febrile period, when the temperature often reaches 107° or 108°F. may be significant taken in conjunction with the fact that multiplication is also rapid in the body of the tick at a temperature about 30° lower. Additional studies on the matrix which seems to bind together the individual *Rickettsiæ*, which compose a colony, might also bring to light information regarding a medium which would justify further work along the lines of artificial cultivation.

Another feature which all pathogenic *Rickettsiæ* possess in common is the habit of becoming parasitic in the vascular endothelia. This is the usual location in human and mammalian tissues of the *Rickettsiæ* of Rocky Mountain spotted fever, in which condition, however, they may spread to the media and adventitia and occasionally to parenchymatous elements such as liver cells.[5] The *Rickettsiæ* of typhus fever are somewhat more restricted, being found only in the mononuclear cells of the perivascular nodules in addition to the endothelium; while the *Rickettsiæ* of heartwater are the most specific of all, since their location is confined, as far as can be ascertained, without exception, to the endothelium.

This restriction of a pathogenic microorganism to a single type of cell has recently been claimed for the tubercle bacillus by Sabin and her coworkers.[6] She believes that the tubercle bacillus attacks a specific kind of cell—the monocyte—penetrates into its interior and multiplies within it. The evidence for intracellular multiplication is conclusive. Maximow[7] has observed the process in the living cells of tissue cultures; but he has found that other cells, as well as the monocyte, are invaded, and some believe that the monocytes do not differ fundamentally from

[5] Nicholson, F. M., *J. Exp. Med.*, 1923, xxxvii, 221.

[6] Cunningham, R. S., Sabin, F. R., Sugiyama, S., and Kindwall, J. A., *Bull. Johns Hopkins Hosp.*, 1925, xxxvii, 231.

[7] Maximow, A. A., *J. Infect. Dis.*, 1924, xxxiv, 549.

the macrophages,[8] or clasmatocytes, as they are often called. The case of heartwater is more concise, for there can be no dilemma in the identification of endothelium. While the *Rickettsiæ* of heartwater may apparently live and multiply within the endothelial cells, there is no evidence to indicate that their action stimulates the endothelial cells to divide and increase in number in a fashion comparable with the influence exercised by ingested foreign materials, such as the tubercle bacilli, upon the monocyte.

It is interesting to note that, although in heartwater the endothelial cells remain living and normal and are so heavily charged with *Rickettsiæ* that they may be considered to be marked by their presence, the free large mononuclear cells of the circulating blood (monocytes, endothelial leucocytes, etc.) which Mallory and his associates believe to be developed directly from the endothelium, remain wholly devoid of *Rickettsiæ*, or at least contain *Rickettsiæ* so infrequently that they have thus far escaped observation.

SUMMARY.

The *Rickettsiæ* of heartwater are more definitely restricted to the vascular endothelial cells of infected animals than are those of typhus or Rocky Mountain spotted fever. They likewise form more pronounced spherical colonies within the cells. Their presence does not injure the endothelial cells to a degree at all comparable with that caused by the other pathogenic *Rickettsiæ*. The rupture of endothelial cells and discharge of *Rickettsiæ*, which are apparently viable, into the circulation constitute phenomena not thus far reported in the case of typhus or Rocky Mountain spotted fever.

EXPLANATION OF PLATE 31.

All the figures were drawn at the level of the table with a 1.30 aperture apochromatic objective of 1.5 mm., compensating ocular 18 and camera lucida. They have been reproduced without reduction so that, as they appear, they represent a magnification of 4033 diameters. They have all been made from tissues of Goat 4510.[9]

[8] Lewis, M. R., Willis, H. S., and Lewis, W. H, *Bull. Johns Hopkins Hosp.*, 1925, xxxvi, 175.

[9] For details of temperature reaction and postmortem examination, see Cowdry, E. V., *J. Exp. Med.*, 1925, xlii, 231.

FIG. 1. Cerebral cortex fixed in Regaud's fluid and stained with eosin and methylene blue. Several colonies of *Rickettsiæ* are seen in the cytoplasm to the left of the nucleus. They are of variable size. In the smallest, a single organism is represented which is slightly larger than the others, is colored rather more intensely and seems to be in the act of division.

FIG. 2. Cerebral cortex fixed in Regaud's fluid and stained with hematoxylin and eosin. The *Rickettsiæ* are feebly colored. They are contained in an unusually large vacuole-like space (distal to the nucleus, *i.e.*, between it and the lumen) the upper margin of which can be clearly distinguished. Through growth pressure they have indented the nucleus.

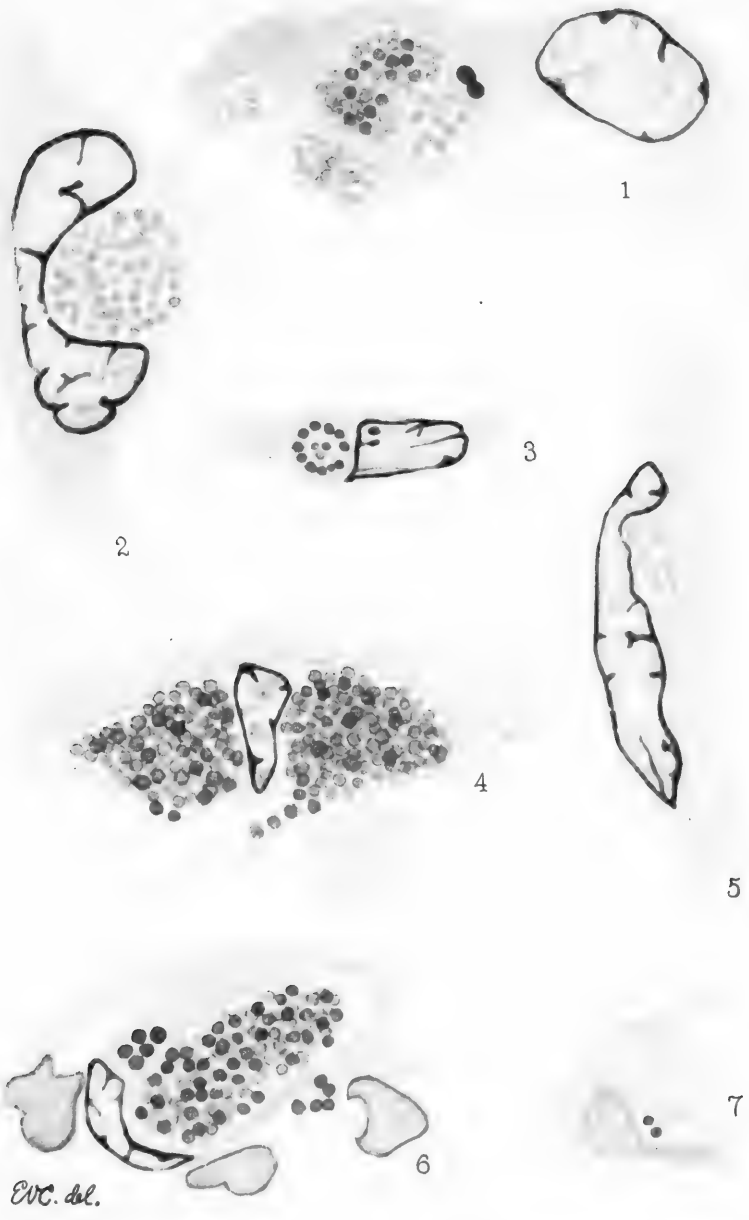
FIG. 3. Cerebral cortex fixed in Regaud's fluid and stained by Giemsa's method. A colony of *Rickettsiæ* is represented at one pole of the nucleus. Organisms which seem to be dividing are oriented with plane of division at right angles to the margin of the vacuole.

FIG. 4. Pancreas fixed in Regaud's fluid and stained by Giemsa's method. There are two colonies of *Rickettsiæ*, one on either side of the nucleus. Their growth has exerted sufficient pressure to cause the nucleus to alter completely its position by projecting directly toward the lumen in a direction parallel to pressure exerted by the blood stream. A few of the *Rickettsiæ* are being discharged into the lumen.

FIG. 5. Cerebral cortex fixed in Regaud's fluid and stained by hematoxylin and eosin. Here the *Rickettsiæ* are multiplying in a very unusual position, proximal to the nucleus and remote from the lumen.

FIG. 6. Cerebral cortex fixed in Regaud's fluid and stained by Giemsa's method. Two colonies of *Rickettsiæ* are illustrated. Those comprising the smaller one are stained more intensely than the others and are noticeably larger. Some *Rickettsiæ* are escaping from the larger colony into the blood stream.

FIG. 7. Cerebral cortex fixed in Regaud's fluid and stained with Loeffler's methylene blue. Two *Rickettsiæ* embedded in some chromophobic material are seen in the lumen between the erythrocytes.



(Cowdry: Etiology of heartwater. III.)

UNIV. OF MICH.

THE RELATIVE REACTION WITHIN LIVING MAMMALIAN TISSUES.

VI. Factors Determining the Reaction of Skin Grafts; a Study by the Indicator Method of Conditions within an Ischemic Tissue.

By PEYTON ROUS, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Plate 32.

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In previous papers from this laboratory a technic has been described for the examination of tissues vitally stained with litmus or with indicators of the phthalein series (1), and some observations on the apparent reaction within various organs have been recorded (2). Recent control tests on the influence of tissue materials upon the colors manifested by phthaleins staining them have shown that these colors are not conditioned to any important degree by "salt and protein errors," but that they are really indicative of the prevailing pH (3). By means of vital staining with selected indicators one should be able to apprehend, and may even come to understand, certain physiological states inaccessible to study by approved quantitative procedures. One such state, that of "outlying acidosis," has already been briefly reported upon (4). The present paper is concerned with the conditions in a tissue, the skin, which survives when its blood supply has been cut off. The problem has both practical and theoretical ramifications. In corollary to it the changes which result from superimposed injuries to the tissue elements will be considered.

The Vital Staining of Skin Grafts with Phenol Red.

Male white mice of 25 to 30 gm. were shaved over the sides and back, from fore legs to hind, and under ether anesthesia pieces of the skin were excised, midway between axilla and groin, and at once sewn back in place again. These pieces were roughly circular and from 1.0 to 1.6 cm. in diameter. They had the entire

thickness of the integument and contained, in addition to epidermis and corium, a varying quantity of fat and the thin layer of striped muscle which in the mouse and rat extends superficially over almost the whole body. It was frequently impossible to remove such large pieces without cutting cutaneous vessels that supplied neighboring regions, and in consequence of this occurrence the grafts often failed to become vascularized promptly and perished after some days. In females the mammary gland proved a serious complicating factor, and after a few trials males alone were used. The best results were had when the graft was not separated all at once from its surroundings but left attached by an isthmus that was severed only after the major portion had been sewn in place. By this procedure the swabbing of the raw surfaces with saline solution to prevent drying was reduced to a minimum; but great care had to be taken in the dissection else some of the loose web of tissue connecting the skin with the underlying parts escaped being cut, with result that total ischemia of the graft did not develop. For the sewing a fine curved needle carrying a single strand of the three which are wound into No. 2 surgeon's silk was employed; and an over and over stitch. Perfect approximation of the wound edges proved essential, for when a part of the wound bed was left exposed necrosis usually spread from it (5). Aseptic conditions were maintained during the operation; and no dressing was put on afterwards. The mouse had to be prevented from removing stitches and graft however. This was accomplished by passing its head through a hole in the center of a flat disc of pasteboard. The projecting collar thus formed, which stood out like the ruff of a clown, did not interfere with the animal's movements but kept it from gnawing at the graft. The stitches were removed on the day following the operation, and thereafter for nearly a week the attachment of the implanted skin was but frail.

Each day the mouse was injected into the peritoneal cavity with 0.5 cc. of a watery 4 per cent solution of phenol red, made as follows: 2 gm. of the phthalein (Hynson, Westcott and Dunning) is ground to a paste with a little water, 9.7 cc. of N/1 NaOH added, and then water to 50 cc. Such a solution is at pH 7.4 (as determined with the potentiometer) and approximately isotonic with 0.9 NaCl. The quantity of alkali employed somewhat exceeds that required, on theory, to bring the indicator to the hydrogen ion concentration mentioned, presumably because of acid impurities in the material. In order to effect the injection into the mouse without a struggle that might have entailed separation of the graft it was briefly anesthetized by dropping it into a jar containing cotton soaked in ether.

During most of the 1st week after implantation the graft appeared pallid,—save when vitally stained,—and bloodless. As is well known vessels begin to penetrate into transplanted skin during the second 24 hours, but there is certainly no effective circulation of blood for a much more considerable period in grafts of the size studied in the

present work. This is evident not merely from the aspect of the graft under ordinary conditions but from the slowness with which it colors up after an intraperitoneal injection of phenol red. On the 3rd day after operation it stains no more rapidly than on the 1st. Thereafter, though, it lags in this respect less markedly as compared with the skin round about, and by the end of a week, in successful instances, it colors as fast as the latter.

The rate of coloration provides enlightening data on the fluid interchange taking place within engrafted tissue. Phenol red is very highly diffusible, coloring mice deeply within a few minutes after an intraperitoneal injection; and the brilliant, ruddy color of the stained animals is due for the most part to an extravascular penetration of the dye. This has been shown by perfusing the stained animal until free of blood, with warm Locke's solution introduced into the beating heart, after the inferior vena cava has been snipped across (6). But the fact emerges even more strikingly from the observations on the skin grafts of the present work. These color deeply, evenly, and surprisingly fast with the phthalein at a time when it is certain no blood can reach them, that is to say within a few minutes after they have been separated and sewn in place again. The hue they manifest is referable to staining of the subepidermal tissue fluids and tissues, especially the corium. Muscle stains but slightly with the dose of indicator I have employed; fat scarcely at all; while the epidermis is so thin and so lightly stained that it may be dismissed from account.

In recording the hues of animal and graft Ridgway's "Color standards and nomenclature" (7) has proved of great service. Wherever the hues provided by this book are mentioned in describing the findings italics will be employed. For the matching, one of the standards at a time was exposed through a hole cut in a sheet of white paper, and compared with the skin color. A normal mouse of 28 gm. given 0.5 cc. of phenol red ordinarily becomes deeply stained within 15 minutes, and reaches a maximum color, one varying between *jasper red* and *eugenia red*, about 30 minutes after the injection. The healthy avascular graft usually remains entirely unstained for from 10 to 15 minutes (Fig. 1) after the injection,—a staring, pallid patch in the midst of the red body surface,—and then it begins to turn yellow here and there. The staining reaches a maximum intensity, *apricot*

orange ordinarily, after a little less than an hour in all, and then appears even (Fig. 2). The hue of many mice will already have begun to fade, owing to elimination of the phthalein into bile and urine; but long after the general decoloration has taken place, a process usually completed by the end of $2\frac{1}{2}$ hours, the graft remains brilliantly tinted (Fig. 3). It is still yellow more than 5 hours after the injection; for the dye leaves the avascular tissue far less rapidly than it enters it.

These are the happenings whenever the animal is stained during the first 3 or 4 days after the implantation,—and it can be stained again and again without evident injury to either it or the graft. Later, as the new vessels become effective and the penetration of the phenol red into and out of the implanted tissue does not lag so noticeably, the color of the graft comes day by day to have less of yellow and more of red in it. By the end of a week the "take" is usually perfect, and the phthalein coloration differs practically not at all from that of the host.

Abnormal Reaction of the Grafts.

The color of the stained graft seems to indicate that it is acid as compared with the normal skin round about; and there is every reason to believe that this is the actual case. Phenol red is a stable indicator, not liable to error through its association with tissue components (8) with the important exception of the proteins of plasma. Kendall (9) states that some destruction of it by reduction occurs within the organism; but the amount changed in this way is negligible when large quantities are given, as was the case in the work here reported. Dr. D. R. Drury, of the laboratory staff, has recovered from the urine of a rabbit more than 96 per cent of the phenol red required to stain it vitally, and a part of the missing 4 per cent was present in the feces, which were not extracted. For nearly 40 years (10) the fact has been generally recognized that acidity develops in tissues when their blood supply is interfered with. If a string is tied tightly around the shaven leg of a rat stained with phenol red the color of the leg turns from red to orange within a few minutes, only to become red again shortly after the cord is loosened and circulation resumed. If one evert a flap of living subcutaneous tissue vitally stained with the dye it turns from

red to purple in proportion as carbon dioxide escapes from the raw surface; and the purple can be converted to orange-yellow by brief exposure of the tissue to an atmosphere of CO_2 (11). These are simple instances illustrating the readiness with which the phthalein reacts under *in vivo* conditions and showing that it reacts characteristically.

One can appraise the color of the stained body surface of the living animal by oiling it, placing over it an Autenrieth wedge filled with water, and comparing the color as thus viewed with that obtained by superimposing a similar wedge, filled with an appropriate buffer solution colored with phthalein, over the oiled and shaved skin of a normal animal. By varying the buffer solution and moving the colored wedge until precisely the right depth of fluid is obtained, one can closely approximate the hue of the stained tissue. *Apricot orange* corresponds with a pH of about 6.8, *jasper red* with pH 7.4, and *eugenia red* with pH 7.6. The actual figures can be disregarded. It is their relation to each other which tells the story. Evidently vigorous skin grafts of the mouse are, relatively speaking, about pH 0.6 more acid than the normal skin, and they are able to survive this state of affairs for several days. The acidity is referable, at least in the beginning, to the elements proper to the tissue, not to the many cells that wander in (12), as is sufficiently shown by its development within an hour after implantation of the graft.

Vigorous Grafts Are Acid, Weak Ones Alkaline.

At an early period in the work, when the operative technic was uncertain, grafts coloring red with the phthalein were not infrequently encountered. It was natural to suppose that these, as manifesting a tissue reaction close to the normal, would be the ones to survive; and not until some thirty orange or red examples had been followed did the fact emerge that it was precisely those grafts which showed for days the orange color of an abnormal acidity that lived and healed into place. Whenever the implanted bit of skin, or a portion of it, repeatedly stained red during the early period after operation when it should have been reestablishing itself, that graft or portion was noted soon after to perish. Once this had been realized reasons for the color difference were not far to seek. Owing to poor fluid interchange the acid products of the cell metabolism of healthy grafts would tend

to heap up, just as they accumulate in subcutaneous areas temporarily deprived of circulation through the vascular contraction following on a local injection of epinephrine (13). The cells of injured tissue on the other hand not only work less actively, or dying, cease to work at all, but to a greater or less degree they lose that semipermeability which characterizes them during life. As result they are penetrated by the alkaline lymph which acts to sweep away such acid products as may arise through autolysis, and, coming gradually into equilibrium with this fluid, they tend to approach it in reaction. One may doubt whether the interstitial fabric which forms so large a part of the corium, though staining deeply with the phthalein (14), possesses life of its own in any proper sense. One must think of its reaction as determined preponderantly by the activities of the cells dispersed through it.

Grafts Injured Experimentally Are Alkaline.

To test this explanation of the findings pieces of skin of the usual size were damaged prior to implantation. The intention was to inflict the minimum insult that would ensure an eventual failure of the graft. Heat was employed in some cases, but repeated freezing and thawing proved better for the purpose.

In order to heat the pieces of skin they were folded upon themselves with the raw surface inwards, and placed far down in test-tubes already in a water bath at 50–51°C. The grafts adhered to the sides of the tubes which had moist gauze at the bottom to prevent drying. They were heated for from 7 to 10 minutes.

When a graft was to be frozen and thawed it was spread upon a sterile mica slide with its raw surface against the latter. To prevent drying the edges were sometimes folded under; but more usually a drop of salt solution, or Ringer's solution, was run around the edge of the tissue. The slide was then placed on the freezing disc of a microtome, the tissue frozen solid by the escape of CO_2, and the preparation removed and thawed at once with the warmth of the hand or that of a metal plate at about 38°C. The processes of freezing and thawing were carried out three or four times as rapidly as possible, and the graft was replaced in position. Meantime the skin defect on the body of the animal was covered with a sponge moist with salt solution or Ringer's solution.

It proved easier to return the grafts to their original position when they were asymmetric or had been cut with a slight projection at one point in the periphery that fitted into a notch in the skin.

Heating to a temperature of 50°C. for 10 minutes should have killed the tissue,

under the conditions. Such heat regularly results in an eschar after 2 days, when applied to the shaved body of rats by way of a glass disc through which hot water circulates. But it was only after the lapse of about a week that the heated grafts became evidently necrotic. Until then they maintained the aspect of life, being pliable though definitely swollen and somewhat more opaque than control grafts on the other side of the same animal. They continued to be pallid, however, long after healthy grafts had begun to show the flush of a renewed circulation; and eventually they dried, remaining adherent over the advancing edge of the skin as it encroached on the defect beneath them.

If the appearance of the heated grafts was for a long time much like that of surviving tissue this was never true of their reaction. From the moment that they were sewn back in place they were always frankly alkaline to phenol red, as alkaline to appearance as the surrounding normal tissues and occasionally somewhat more so, being then of a more purple hue (Fig. 2).

Repeated rapid freezing and thawing, unlike heating, failed to render a graft more opaque than ordinary, and when first sewn in place it had precisely the appearance of the control graft on the other side of the same animal. Yet freezing and thawing is known to kill mammalian cells of many sorts; and I have repeatedly utilized it to destroy the cells of a transmissible chicken sarcoma without injury to the filterable agent responsible for the growth. It did cause eventual failure of the skin grafts. These retained their appearance of life for a week or 10 days, though, and often developed what appeared to be a surface union with the surrounding tissues. They never vascularized, however, but remaining pallid, and, becoming gradually thinner and parchment-like, they dried up. Unlike heated grafts they were never in the least edematous. From first to last the frozen and thawed skin stained red with phthalein, often a more purple-red than the animal.

It was, as has been stated, a part of the plan of the experiments to injure the implanted skin only to the extent necessary to insure a failure to "take." For, obviously, greater tissue changes would have lessened the chance that the graft would remain placed and in condition to take the stain, and have complicated the interpretation of the results. That the heating was close to the critical amount appears from the fact that a graft submitted to only 48°C. for 7 minutes

stained *apricot orange*, as does a healthy graft, immediately after it has been replaced in position, *coral red* 24 hours later, and it only attained to the *jasper red* of the surrounding body surface on the 2nd day after operation. The long persistence of the frozen and thawed grafts and their temporary union with the surroundings also bespoke the minimum damage compatible with the purpose in hand.

The experiments support the view that the orange staining of the grafts destined to "take" is referable to acid products arising out of the life processes taking place within them, and that the ruddy hue of grafts doomed to fail results from an impairment of such processes.

The Altered Permeability of Injured Grafts.

In not a few instances the avascular implants injured by freezing colored up more rapidly with phthalein than did control implants on the opposite side of the animal (Fig. 1). The latter, though, were somewhat edematous which might well have interfered with the fluid exchange; while furthermore the orange color of the phenol red penetrating into them was not so readily to be perceived as the purply rose in skin that had been frozen and thawed. For these reasons the difference in the rate at which the grafts stained could not be certainly ascribed to a loss of semipermeability of the injured tissue; and it was necessary to resort to special experiments, now to be described, to determine whether such a loss had occurred. The injured grafts retained phenol red for quite as long a period after decolorization of the host (Fig. 3) as did their healthy fellows—a fact which would indicate that the acidity developing in the latter did not lead to any unusual fixation of the dye upon them, like that encountered by Kendall in another relation (15).

For the tests of relative cell permeability in healthy and injured implants erythrolitmin has been employed. The color of animals vitally stained with this indicator is due in the main to an intracellular storage of it; and through local changes in the hue one is enabled to perceive at once when the barrier of semipermeability normally maintained by the cells is broken down. The highly diffusible phenol red is not segregated within cytoplasm, under ordinary conditions at least, and hence it could not be utilized for the work.

Rats and mice repeatedly injected with litmus, or with erythrolitmin, the effective constituent of Kahlbaum's cube litmus as at present available, have much of the indicator segregated within the cells of the subcutaneous tissue, though there is also some diffuse distribution of it throughout the intercellular material. The diffusely distributed litmus is always in the alkaline or blue form, whereas the segregated is red (16). As result of a combination of the two sorts of staining the living animal appears violet-pink for some weeks after injection. Everywhere throughout the corium one finds microscopically that the cells are crowded with pink or red globules, while the intercellular fabric has an even, light blue tint. Injury to the individual cells results in an immediate color change of the globules from red to blue, and the litmus diffuses from them secondarily, staining the cytoplasm and nucleus blue (17). A similar secondary staining under such circumstances with stored vital dyes having no indicator properties has long been familiar to cytologists; and it is plainly referable to a loss of the normal semipermeability. The change from red to blue of the litmus-containing intracellular granules which occurs prior to diffusion of the dye from them is traceable to a like event, the acidity that had prevailed within the segregated material being lost as the alkaline tissue fluids penetrate to it through the cytoplasm. Any gross trauma to the skin of the stained and living animal results at once in a change in the color of the region affected from violet-pink to a sharp blue.

For the purpose of the present tests mice were three times injected into the peritoneal cavity at intervals of a day or more with 0.35 cc. of a 2 per cent solution of erythrolitmin in 0.9 per cent NaCl.[1] Between 1 and 2 weeks later skin grafting was carried out, according to the accustomed technic; but the graft was frozen and thawed three or four times before it was sewn into place. In several animals a control graft of the same size was removed from the other side and replaced at once.

When taken for freezing the graft had the same color as the rest of the integument—a hue approximating the *light vinaceous lilac* of Ridgway (Fig. 5). But immediately that it had been frozen and thawed it changed to a brilliant blue, *Blanc's blue* or *Yale blue* (Fig. 5); and this hue it retained as long as it continued to fill the skin defect. This it did for the period usual with such frozen and thawed material, remaining flexible, and translucent for a week or more, during which period a smooth union with the neighboring skin often appeared to take place.

Despite the most careful handling the healthy graft placed on the other side of the animal also changed somewhat from the hue it had prior to separation. Wherever it was held with forceps or thrust through in the course of the suturing it became blue, as did also the normal skin so treated; and in addition, after it was sewn back, it was noted to be somewhat bluer throughout than its surroundings were (Fig. 5). Next day this unusual color was still as pronounced as at first, and

[1] The erythrolitmin was prepared by a modification of the older methods, which will be described by Dr. P. D. McMaster in a forthcoming paper.

only toward the end of the 1st week, as the graft became established, did the hue gradually revert to the "normal."

The expectation had been that the blue erythrolitmin liberated in the frozen and thawed graft by death of the cells would be carried off little by little in consequence of the local fluid interchange, and that in consequence the color would become much lighter. But, as just mentioned, even the relatively slight amount of blue pigment liberated in the healthy graft persisted as such in it for several days. Erythrolitmin has an affinity for intercellular substances, which remain blue for months after the coloration has disappeared elsewhere (18); and it is highly colloidal, passing to and from the tissues with difficulty. In the light of these facts there is no need to invoke chemical alterations in the fabric of the several times frozen graft to explain its enduring blue color.

The observations demonstrate that the cells of grafts frozen and thawed repeatedly lose their semipermeability. That the change takes place everywhere and all at once may be doubted however: for not only do the grafts retain the aspect of life for many days but their color by transmitted light, prior to implantation after the injury, is ruddy here and there, showing that some of the erythrolitmin still persists in the red form. Only by reflected light is the hue a deep, clear blue.

Permeability of Skin Grafts for Carbon Dioxide.

The increased permeability of the damaged, avascular skin graft has been shown in another way, namely by submitting the body of a mouse carrying it and a healthy graft to an atmosphere of carbon dioxide. The gas penetrates the injured skin with immense rapidity, rendering it acid.

It was Lavoisier himself who first showed that carbon dioxide passes out of the intact skin of mammals; and his observations have been often repeated, with variations. When the skin is hyperemic and moist a not inconsiderable gaseous interchange may take place through it, as much as 4 per cent of the total CO_2 being eliminated in this way (19). To the present no experiments seem to have been made on the penetration of CO_2 from without. Skin grafts *in situ* are ideal for a study of the phenomenon since their avascular condition creates an opportunity for the gas to accumulate within them, as it cannot to any considerable degree within the normal integument.

For the purpose of the tests there has been utilized the funnel gas chamber devised for a study of the changes in pH occurring in raw tissue surfaces (20). The CO_2 was let in at *D* in the apparatus as figured in the paper referred to, and

it escaped, in some part, through the other tube *E*, which had now been provided with a connection leading off to the floor. The mouse lay on top of two layers of rubber dam which in turn were spread upon an electrically heated pad. The animal had been stained with phenol red as usual and rendered quiet by the intraperitoneal injection of 0.15–0.2 cc. of 20 per cent urethane shortly before the observations were begun. These latter were only undertaken 2 days or more after the body surface of the animal had been shaved and the grafts placed, in order to rule out the possibility of any entrance of the CO_2 through surface abrasions. The hair had been removed from about the neck of the animal with a sodium sulfide solution; and a round hole in the rubber dam closing the gap at the margin of the funnel fitted the neck snugly. The edges of the dam were everywhere attached to the funnel by adhesive except below, where the rubber extended along the pad in a broad apron ending 6 to 10 cm. away from the head of the mouse. In order to rule out any possibility that the animal might inhale CO_2 a second piece of rubber dam was placed about its neck, over the first, to block off the gas chamber more completely; and the head was thrust into a small funnel through which a continuous gentle stream of air was blown against the nostrils. Tests with smoke after the experiments showed that some CO_2 escaped from beneath the funnel here and there, as well as through the proper outlet for it, but that none whatever could have been inhaled. A thermometer was introduced into the gas chamber through separate piercings in the layers of rubber dam, with the bulb lying between the groin of the mouse and the heating pad. The temperatures ranged between 36° and 38°C.

When an atmosphere consisting entirely of CO_2 was to be used the gas was led in from an ordinary Kipp generator after passage through a wash-bottle. The water in this latter never contained more than a trace of HCl, and there would seem to be no possibility that the effects on the skin surface of the mouse can have been due to another cause than carbon dioxide. The rapidity with which they disappeared when the surface was once again exposed to air also bespeaks the action of the gas. In some special experiments a mixture from a compression cylinder, containing 10.35 per cent CO_2 and approximately 20 per cent O_2, and 70 per cent N_2 was employed. Dr. C. A. L. Binger kindly determined the percentages.

When comparisons were to be carried out with the Ridgway color standards the stopper was briefly removed from the top of the gas chamber so that the inspection could be made without the intervention of a glass wall. Access to the body of the mouse could also be had in this way without changing the gas. To replace the latter with air a tube was thrust in and the chamber emptied almost instantaneously with the aid of the laboratory vacuum.

On running pure carbon dioxide into the chamber a change could be noted practically at once in the color of the frozen and thawed, avascular graft. It began to turn from *eugenia red* through *apricot orange* to a brilliant untempered *orange*, reaching this hue within 8 or 10 minutes (Fig. 4). Further exposure to

CO_2 did not alter the color for the good reason that the acid end of the range of phenol red had been attained. The control graft on the opposite side of the animal was *apricot orange* or *apricot buff* to begin with; and during the brief period in which the frozen and thawed tissue was running the gamut from purply red to orange it altered slightly, to *zinc orange;* but it obviously lagged in changing color as compared with the injured tissue and was still definitely less orange than the latter after 15 minutes, the maximum period of the observations (Fig. 4). On the other hand when once again exposed to the air it kept its unusually intense orange color long after the frozen and thawed skin had once again become *eugenia red*. This happened within about 15 minutes.

During the time that the grafts were undergoing these changes a slight but definite alteration was to be noted in the color of the body surface generally. It turned from red toward yellow, that is to say from *eugenia red* to *jasper red* in the case of animals approximating the first mentioned hue, and from *jasper red* to *carrot red* in some other individuals. There is, by the way, not a little variation in the surface hue of normal animals stained with phenol red, as the mention of these differing initial colors will attest. Variations in the normal pH of the blood, similar in magnitude to those here indicated by the phthalein have, of course, long been recognized to exist.

In order to determine whether the general change in color of the animals submitted to CO_2 was referable to absorption of this gas by way of the grafts or to a passage of it through the undisturbed skin everywhere, normal mice were shaved from fore legs to hind and 2 days later were exposed to pure CO_2. There ensued changes in the general hue identical with those just described. At the end of three-quarters of an hour they were no more marked than after 15 minutes. They were indicative of an apparent fall in pH from about 7.6 or 7.5 to 7.4 and 7.3 respectively. The rapidity with which the color reverted to the "normal" when the mouse was once again exposed to air was startling. Within 3 minutes the change had been completed. The surface acidosis described evidently resulted from a continuous passage of CO_2 through the skin, one not entirely compensated for locally, in the surface regions at least, by circulatory and respiratory readjustments. In a number of animals the rate and amplitude of the breathing were followed throughout the experiment. Changes accompanied the exposure to CO_2, and doubtless analyses of the expired air would have shown that no inconsiderable quantity of the gas, in addition to that resulting from body processes, was being given off through the lungs.

Water never condensed within the funnel chamber out of the CO_2; and the skin of the mice was as dry to the feel as ordinary. The findings certainly cannot be laid to the presence of an abnormal amount of moisture on the skin surface. Moistening the grafts led, as was to have been expected, to a more rapid penetration of CO_2 into them. To demonstrate this drops of distilled water were placed here and there on the skin and on the grafts before the gas was run in. They stood high and hemispherical, like dewdrops; and after various periods were

removed. It was possible to do this with no other alteration of conditions within the chamber than were involved by the introduction from above of a long-handled forceps carrying a piece of filter paper to blot up the fluid. At times when the injured graft was becoming acid in consequence of the exposure to CO_2 but had not yet attained to the hue of *orange* a narrow circle of this hue could be briefly seen after the application of the filter paper, a circle corresponding in situation to the edge of the drop, the place at which the layer of water had been thinnest. When now the graft was reexposed to air the regions still moist turned purple first. Healthy grafts gave less outspoken findings; and only occasionally were slight differences of the general nature of those just described to be seen on the intact skin.

The experiments brought out a number of facts. Carbon dioxide penetrates the intact skin of the shaved mouse so rapidly as to cause some change in the surface hue of animals stained with vital red; it renders vigorous skin grafts somewhat more acid than they already are as the result of ischemia; and it penetrates injured grafts with an astonishing rapidity, rendering them pronouncedly acid. How acid the injured tissue can become is not yet certain, for no indicator other than phenol red has been employed.

Even in vigorous skin grafts there is much cell degeneration and death. The epithelium in particular is soon reduced to a thin layer of living cells (21). When the tissue has been injured experimentally the retrograde changes must be still greater; and one might think of the heated, or frozen and thawed, graft as a mere raw dead surface exposed to the air, did not its texture and the absence of seepage or drying belie the view. Doubtless the alkaline reaction of grafts thus injured is due in some part to an escape of carbon dioxide from them. The experiments involving exposure for a long time to a gas mixture containing 10.35 per cent CO_2 possess significance in this connection. Exposure to such a proportion of CO_2, approximately twice that in alveolar air, leads to an alteration in the hue of injured grafts,—there is a change from *eugenia red* (or purpler) to *carrot red;* but the alteration is slight, as compared with the change to *apricot orange* promptly undergone by stained pieces of healthy skin when grafted in an atmosphere of air. Exposure to 10.35 per cent CO_2 does not result in any definite color change in the mouse's body surface generally, and leads to only dubious ones in healthy grafts already *in situ*.

The Association of Tissue Acidosis and Edema.

Healthy skin grafts are regularly somewhat edematous during the first few days after implantation, at the time that is to say when the local reaction is acid as compared with that of the rest of the integument. A similar association of edema with tissue acidosis is not infrequent under other circumstances,—prevailing opinion and potentiometric determinations to the contrary notwithstanding. It can be observed to exist about surface abrasions in animals vitally stained with phenol red, as well as at other points of local inflammation, the phthalein in the swollen areas being orange as compared with the red of that in the normal skin nearby. And a rapid local development of edema and acidosis takes place when a tube through which water circulates at 50–52°C. is applied to the skin of an anesthetized rat stained with phenol red. Under such circumstances the edema and a change from red to orange of the phthalein contained in the region involved by it both become pronounced within 15 minutes.

In contrast to the swelling and acidity manifested by healthy grafts one sees in frozen and thawed grafts not the least edema, and a reaction which is definitely alkaline. The facts are not to be taken, however, as furnishing support to the hypothesis that acidosis determines edema. Edema develops, yes, in the living and acid skin graft, but it progressively disappears during the days immediately after the grafting, whereas the acidosis does not diminish. Furthermore edema is regularly to be met in grafts that have been injured by heat, although the reaction of the injured tissue is pronouncedly alkaline. In a succeeding paper observations are reported which would suggest that under the conditions of widespread and enduring tissue acidosis brought about with hydrochloric acid no important water retention occurs.

DISCUSSION.

The foregoing observations provide data on the conditions which prevail in skin grafts and determine their survival; but it is in a broader relation that they have principal claim to attention,—namely, in relation to the happenings within ischemic tissues as a class. Despite the general recognition that interference with the blood supply

of a living tissue results in the local formation of acid substances the corollary that ischemic tissues must endure for some time in an acid milieu if they are to survive seems not to have had the attention that it merits. As the present work shows, the more vigorous the cells the more acid do they render the tissue when its blood supply is interfered with; and the tissue survives despite this acidity. Skin has a relatively slight metabolic activity, and some part of the carbon dioxide accumulating in avascular grafts of it must escape to the air or into the body of the animal, while a continuous, if slow, fluid interchange with the neighboring tissues acts also to reduce the local acidity. Yet notably acid the tissue is, nevertheless. This being the case what must one suppose the reaction to be within a leg severed and reunited without suture of the vessels (Halsted), or in an arm surviving despite a clot in the axillary artery? Can one doubt the development of a more pronounced acidity in these ischemic members?

That cells of some sorts will survive and proliferate *in vitro* in a frankly acid medium is a fact sufficiently attested (22). But the occurrence of proliferation renders the case somewhat different from that of tissue surviving within the body under acid conditions. For it might well be that the individual elements of a culture tolerate the condition of acidity but poorly, and that the strain survives only because its elements continue to divide, furnishing fresh entities more rapidly than the injurious medium kills them off. An analogy to this state of affairs is to be found in the growth of certain tumors composed of cells surviving for so short a time that retrogression of the mass would inevitably ensue were not the rate of wastage more than outstripped by that of proliferation. The condition is one familiar to every student of neoplasms. It is often plainly evident when "Chicken Tumor I,"—a transmissible sarcoma,—is cultivated *in vitro*.

No attempt has been made in the present work to determine more than approximately the degree of acidity developing in the grafted skin of the mouse. Manifestly the knowledge could have only a special interest; for one would expect a much greater acidity to develop in tissues of high metabolic activity. To determine the relative reaction within ischemic portions, living and dead, of organs which appear to be frankly acid under normal conditions,—the liver and pancreas for example,—will be an interesting task for the future.

Current generalizations on the changes which take place within tissues dying in the body are largely based upon studies of material

autolyzing in the mass *in vitro;* and perhaps they can be safely applied to large masses autolyzing *in vivo.* The interchange of material with the body round about will have little significance for the immediate fate of large infarcts and accumulations of pus despite the unusual permeability of dead tissue. But when the necrotic mass is small the factor of interchange assumes great importance, as the experiments with the skin grafts show. The occurrences subsequent to the injury of small cell aggregates cannot be explained on the basis that a local accumulation of acids determines autolysis or atrophy (23), for the sufficient reason that the local reaction will tend toward alkalinity rather than toward the acidity of vigorous tissue surviving an ischemia. It follows that the chemical changes which take place in small necroses must differ in some respects from those occurring in large masses of dead tissue. A single set of generalizations as concerns autolytic processes will not cover both instances.

While the life processes of vigorous tissue suddenly rendered bloodless act to create a milieu that would seem prejudicial to survival, the alterations that take place in injured tissue would appear superficially to favor this event. To judge from the observations on skin the lessened cell activity consequent upon injury results in a smaller accumulation of acid material,—save for that referable to the trauma itself (as in muscle); and the increased permeability of the damaged tissue results in a more rapid escape from it of carbon dioxide and of the other substances causing acidity. But needless to say the rough correspondence thus brought about between the reaction of the injured graft and the normal tissue surrounding it is a superficial phenomenon, not the sign of a good state of affairs but of one which masks profound cytological derangements.

The eventual drying of grafts which fail to "take" is not due to any failure to obtain fluid from underneath, as the experiments have shown. Rather must one think of it as consequent on an abnormal loss of fluid from the surface, itself a manifestation of the increased permeability which can be demonstrated by exposure of the tissue to carbon dioxide. There is good reason to suppose that the reaction of the tissue involved in skin lesions often deviates from the normal owing to the influence of the factors dealt with in the present work.

The observation that edema and tissue acidosis sometimes coexist

would at first sight seem difficult to reconcile with the fact that edema fluids as obtained for potentiometric examination are regularly alkaline (24). But the contradiction is merely apparent. Local acidosis and edema occur together only when the fluid accumulation is not very pronounced and the metabolic activities of the tissue are either abnormally heightened,—as during inflammation,—or are taking place under conditions which permit of an accumulation of acid products,—as in skin grafts. There is little doubt that the development of edema can act to maintain alkalinity, the profuse alkaline fluid deriving from the blood having effect to drown out, so to speak, what might otherwise be a local acidosis. I have never found a very pronounced inflammatory edema that did not yield a fluid alkaline to phenol red, although inflammation as such conduces to local acidosis. And Henning (25) who injected N/10 HCl into the leg muscles of guinea pigs, observed that the initial acidity was supplanted after 24 hours by a pronounced local edema and alkalinity. It is conceivable that sometimes during the development of an inflammatory edema there may be such an escape of blood protein into the tissues as will suffice to influence phenol red, with result in a greater alteration in the color of the phthalein than the actual acidosis would warrant. No such happening can be invoked to explain the case of edematous skin grafts, however.

Some comment is necessary on the difference between the hue of the skin surface of the vitally stained mouse, as seen by reflected light, and the color of the corium—the skin component principally stained with phenol red—when viewed by transmitted light. The purply red of the former corresponds with pH 7.5 to 7.6, as ascertained by the wedge method described in the present paper; whereas the connective tissue of the corium, when examined separately under oil, has the yellow-pink of about pH 7.2 (26). The reasons for this difference—one which persists when the blood vessels have been flushed out—are largely to be found in the differing optical conditions, but also in some part in the suffusion of the tissue with an alkaline lymph, heavily charged with phthalein, and in consequence ruddy. When a flap of the oiled skin surface of a deeply stained mouse is compressed between slides so that the interstitial fluid is forced out of it for a moment its color is altered from yellowish red to orange.

The observations here set forth may, perhaps, be thought of as the first steps in an analysis of the physical factors which act to determine the fate of engrafted tissues. It is habit to suppose that this fate depends on the ability to survive temporary ischemia, on absence of infection, prompt vascularization and, in the case of isografts, on a tolerance by the host of the strange tissue, and by the graft of its alien surroundings. So of course it does. But the first requisite for survival, namely the ability to survive ischemia is directly referable to physical conditions within the graft, as is also, doubtless, the development of the vasculature that will eventually rescue the tissue from its precarious state.

Gesell has recently brought forward (27) a theory of respiratory control based on the assumption that changes in the hydrogen ion concentration of the respiratory center are the responsible influence rather than changes within the blood. The happenings within skin grafts furnish a suggestive analogue to what goes on within the center according to the view of this author.

SUMMARY.

By means of vital staining with indicators a study has been made of the changes in reaction and in certain other attributes of a tissue abruptly rendered ischemic. Grafts of mouse skin have been employed as test material. It has been found that almost at once after implantation vigorous grafts become notably acid as compared with the normal skin and that they survive and "take" despite the acid condition, which remains at a maximum for several days. Weak or injured grafts on the contrary tend to be as alkaline as their surroundings, if not more so. Through experiments directed to the purpose reasons for this difference have been found in the lessened metabolic activities of the cells of the injured skin, and in an increased permeability which leads to a generalized suffusion of the damaged tissue with the alkaline lymph and an abnormally rapid escape of carbon dioxide from it. The influence of these factors to determine the reaction of tissues dying within the body has not been sufficiently taken into account in considering the chemical changes that occur after cell death, and some revision of current views regarding these as they affect small necrotic masses would seem called for.

Carbon dioxide penetrates so readily into the living skin as to cause some local increase in the hydrogen ion concentration within cutaneous regions exposed to an atmosphere of it, even when the local circulation and the ventilation by way of the lungs have not been interfered with. It penetrates injured skin with especially great ease.

Tissue acidosis and edema not infrequently occur together; but no relationship between them of cause and effect has been made out.

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EXPLANATION OF PLATE 32.

FIGS. 1, 2, and 3. Right and left sides of a mouse, to show the course of the vital staining of grafts with phenol red. Period: any time during the first 3 days after implantation.

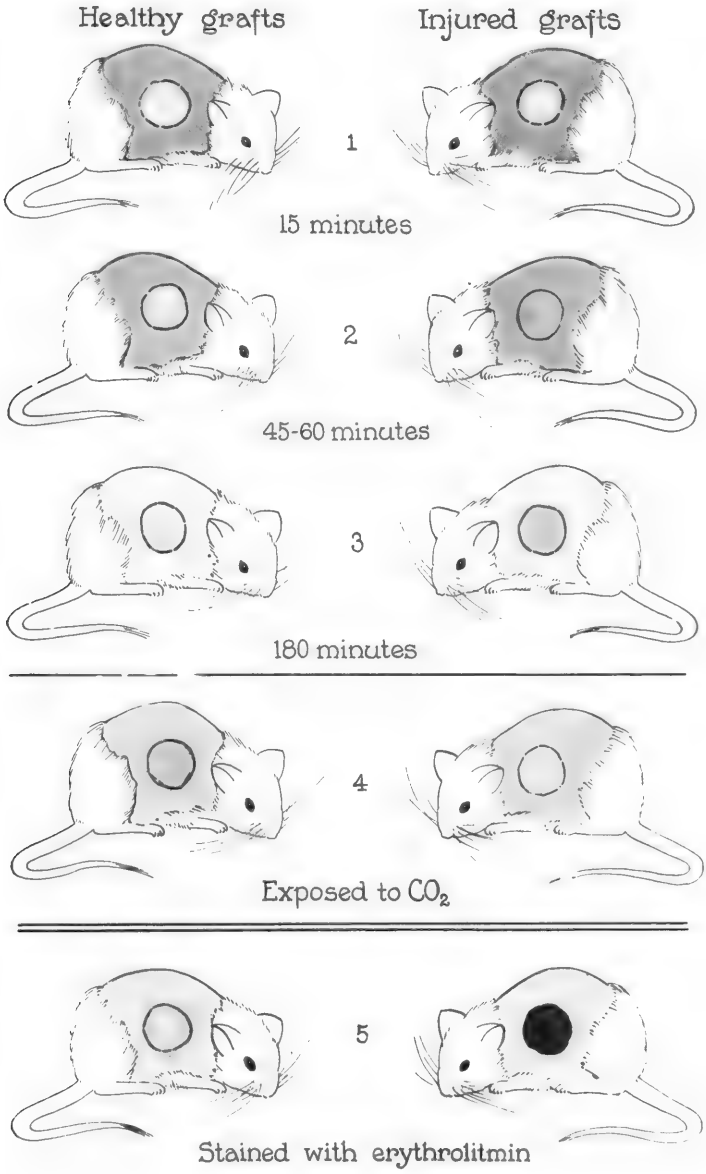
FIG. 1. The staining 15 minutes after an intraperitoneal injection of the indicator. The healthy graft shows no color as yet, whereas the one that was frozen and thawed has become pink. The animal is already deeply colored.

FIG. 2. 45 to 60 minutes after the injection. The grafts are now intensely stained, the hue of the healthy one being indicative of a condition of relative acidity, whereas that which has been injured would seem to be slightly more alkaline than the normal tissue round about, judging from its color. The mouse itself is beginning to decolorize.

FIG. 3. 3 hours after the injection. The animal is now completely decolorized but there is still much phenol red within the grafts and the same differences are visible in them as before.

FIG. 4. Effect on the phenol red coloration of exposure of the body surface to an atmosphere of carbon dioxide for 15 minutes. The injured graft is now no longer alkaline but frankly acid, as evidenced by its hue; and the healthy graft while slightly more acid than ordinary is not as acid as the injured graft. The color of the body surface generally is indicative of a slight change in the direction of acidity.

FIG. 5. Right and left sides of a mouse stained with erythrolitmin, to show the alterations in color of engrafted skin and of skin that has been purposely damaged as well as engrafted. The healthy graft is slightly bluer than the body surface generally, a fact which attests to some injury to its cells with a loss of the normal semipermeability; and the graft which was frozen and thawed is deep blue,—evidence that the cells have become freely permeable to the alkaline body fluids.



(Rous: Relative reaction within living tissues. VI.)

UNIV OF MICH.

THE RELATIVE REACTION WITHIN LIVING MAMMALIAN TISSUES.

VII. The Influence of Changes in the Reaction of the Blood upon the Reaction of the Tissues.

By PEYTON ROUS, M.D., and W. W. BEATTIE, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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The blood is all things to all tissues, and in consequence it can scarcely be expected to manifest the peculiarities of any one of them. Special characters of its own it must have to serve so widely as it does. These elementary principles are often overlooked by those who would speculate upon hydrogen ion concentrations within the body; and one finds not infrequent a disposition to suppose that the tissues and blood must have the same reaction. In several previous papers from this laboratory evidence has been produced that the hydrogen ion concentration within the individual organs differs somewhat (1). In this respect the tissues would appear to be related to the blood as to a sort of physiological base line, from which they stand off at various distances along the ordinate of hydrogen ion concentration. When one produces alterations in the level of the base line, as in the experiments now to be described, the position of many tissues along the ordinate alters also; they become relatively more or less acid or alkaline in rough correspondence with what happens in the blood. Other tissues however, as we shall show, retain their apparent pH unchanged even when the amount of acid or alkali introduced into the animal is sufficient to cause death.

The accumulated data with regard to the various offscourings of the organs as they appear in the blood and the excreta have furnished material for many inferences upon tissue states. But these have remained inferences at best, subject to modification with each new parcel of facts discovered. There have been few attempts to study the reaction of the tissues directly save by quantitative methods

the practice of which involves distortion from the conditions of life. Mention should be made, however, of the interesting experiments of Stieglitz on the reaction of the living kidney as affected by the administration of acid or alkali (2).

Method.

For the present work rats vitally stained with phthaleins were employed; and the living tissues were examined under circumstances which would seem to exclude the ordinary errors of interpretation.

A pair of male rats, from the same stock and of the same age and weight, preferably not full grown, were used in each experiment. They were given no food on the morning of it. The hair was removed from the chest, abdomen, and sides a day or two beforehand in order that any inflammation due to the shaving might have cleared up. On beginning the experiment each animal was given 20 per cent urethane (3) subcutaneously into the back of the neck, in the proportion of 1.1 cc. for every 150 gm. weight, and some minutes later the phthalein into the peritoneal cavity. After ½ hour or more, when the general staining was pronounced, the animals, by now unconscious, were submerged to the muzzle in warm paraffin oil in glass dishes placed side by side under identical conditions of lighting, and after brief preliminary observations the injection of acid or alkali into a jugular vein was begun.

The rectangular Pyrex dishes had a layer of solid paraffin covering the bottom, with a depression for the urine, and they held sufficient liquid paraffin oil—cleansed, and inert to phthalein—to cover the rats. The oil was kept warm by the heat generated when a current was run through coiled "Nichrome" wires surrounded by oil and encased in glass tubing bent to conform to the shape of the dish (Fig. 1). The heating was regulated by means of a rheostat. To distribute it evenly the oil was stirred at intervals with a spatula. The temperature in the two dishes was maintained between 38° and 41°, as nearly the same in both as possible. The legs of the rats were kept in extension by means of long pins thrust through the skin of the paws and into the paraffin bed, while their muzzles were held above the surface of the oil by a thread carried through the skin of the nose and tied about a knitting needle placed across the dish from side to side. Before the oil was poured on, a fine cannula was inserted into a jugular vein of one of the animals, and connected with the apparatus for the injection of the acid or alkaline solution. The other animal served as control. A special arrangement was devised

(3) The influence of urethane upon the blood reaction of rats is known, thanks to the work of Hawkins (Hawkins, J. A., and Murphy, Jas. B., *J. Exp. Med.*, 1925, xlii, 609). For 3 hours or more after injection it causes little change but then there ensues an alkalosis. Our experiments were completed within the initial 3 hours.

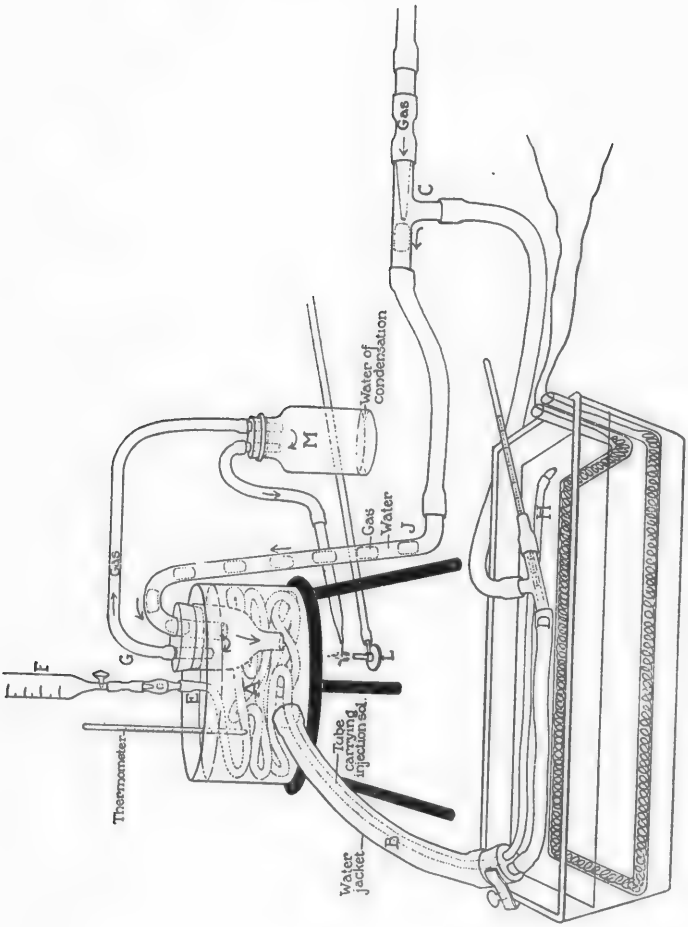
Gas
C
Water of condensation
M
Gas
Water
J
H
Gas
G
F
E
A
B
D
L
Thermometer
Tube carrying injection sol.
Water jacket
B

FIG. 1.

to deliver the injection fluid at body temperature (Fig. 1). The nozzle of a calibrated burette (*F*) which served as a reservoir was connected with a drop gauge (*E*) and this in turn with a long rubber tube coiled in a bath containing hot water at a constant temperature (*A*). The tube passed out through a side arm from the bath, and was encased, as far as the oil bath, in a much larger tube (*B*) containing some of the same warmed water that was in the bath. In order to ensure a circulation in this elongate jacket its further, stoppered end was equipped with an exit pipe (*D*) for the water, in addition to the opening for the rubber tube carrying the injection fluid (*H*). The water was kept flowing through the jacket and back into the bath by the introduction of a slow stream of illuminating gas through a side arm (*C*) of the exit pipe. The passage of bubbles of gas along the pipe (*J*) acted to move water back to the water bath. The gas was delivered separately to be burnt beneath this latter. The water of condensation from the gas collected in the bottle (*M*). For the idea embodied in the apparatus we are indebted to Dr. D. R. Drury.

The tube carrying the solution to be injected now passed beneath the surface of the oil and laterally along the dish,—where its temperature was measured with a thermometer let into the system,—and so to the animal. Near the cannula there was a side arm,—which is not figured,—to permit an adjustment of the pressure immediately after connection with the jugular had been made. Otherwise, when the clamp was taken from the vessel prior to the injection there was sometimes a sudden gush of fluid into the blood with death at once. To prevent blood from passing back into the cannula the latter was turned at an angle to the course of the vein so that its wall would act as a valve across the opening in the glass.

The intensity of the staining of paired rats injected with an equal dose of phthalein was frequently found to differ somewhat; and when phenol red had been used the surface hue often differed slightly as well, variations from *eugenia red* to *jasper red* (4) being observed. The differences in extravascular pH, indicated by these differences in hue, are no greater than the long recognized individual differences in pH of the normal blood. In order to minimize such errors as might be introduced by them when the tissues of the injected and the control rat were compared, several individuals of the same size were often given phenol red at one time and from these the two were selected that most closely corresponded in the character of the staining.

As many of the tissues were examined *in situ* as possible. Since skilled assistance was available the examinations could be made very rapidly. The color of the body surface generally, and of the paws, ears, and mucous membranes, gave

(4) *vide* Ridgway, R., Color standards and nomenclature, Published by the Author, Washington, D. C., 1912. Our method of utilizing this book has been described in a previous paper (Rous, P., *J. Exp. Med.*, 1926, xliv, 815).

direct indications of the reaction of lymph, connective tissue, and cartilage. By a bloodless incision the tendon could be exposed, as too the lymph node of the groin, and the voluntary muscle and the cartilages of the thorax. But the colors in general were most truly seen when the tissues were viewed by transmitted light. This was possible without disturbance, in the case of the connective tissue of a skin fold, and of the cartilage of the ear. Small bits of the other organs were snipped off, and, still surrounded by oil, were examined between mica slips, those from the control and the experimental animal being studied side by side. Glass slides could not be used because of the alkali deriving from them (5). A special plan was followed in taking the specimens to rule out the possibility that the differences noted were due to supravital changes. Thus, when an animal had been injected with acid the bit from it was removed for inspection only after that from the control had been secured,—since the bit longest out of the body would tend to be the more acid as result of the longer asphyxia from interruption of the circulation. If now under these circumstances the tissue fragment just removed from the injected rat appeared more acid than that from the control there was good reason to suppose that a real difference in reaction had existed within the body. Sometimes, to obtain slices of the parenchymal organs, Valentine knives were employed, a different one for each animal, and the cutting was done under oil, on a paraffin base. Bleeding from severed vessels was prevented with mosquito forceps; and cleansed and dried instruments were employed with each specimen. Special care was necessary to avoid contamination with the blood, which, having received directly a greater or less quantity of the acid or alkaline solution, was, of course, altered thereby. In the cases of the parenchymal organs such contamination inevitably occurred, but fortunately it was the tissues of just these organs that remained uninfluenced in reaction by the injections. Cartilage, connective tissue, and tendon could readily be obtained blood-free. It is possible to procure preparations containing all three of these tissues and bone as well from the tip of the tail of the young animal (65 to 90 gm.). This is snipped off with scissors, gently pressed between folds of washed gauze to drive out the blood, and the bony core is rapidly extracted under oil and mounted between mica slides for inspection by transmitted light. By properly timing the procedures one can compare the experimental specimen directly with that from a control treated in precisely the same way and after the same brief interval of separation from the body. Bone and voluntary muscle were not satisfactorily stained under the circumstances of the experiments,—which usually involved a considerable diuresis with color loss,—nor was the epidermis stained nor the splenic substance. A study of the stomach, intestines, and lungs was not made. To all practical purposes, then, the observations were limited to the superficial color, to the hue of the tissue of the ears, *in situ* or on dissection, to connective tissue and cartilage as such, tendon, liver, pancreas, kidney, and superficial lymph nodes. When dissolved in fat the phthaleins no longer act as indicators, though they do in the adipose tissue itself.

The phthaleins employed were cresol red, phenol red, and brom cresol purple. They were employed in watery solution isotonic with the blood and at pH 7.4 except where otherwise stated. The staining was at a maximum when the injection of acid or alkali was begun, practically all the phthalein having been absorbed from the peritoneal cavity. Brom thymol blue could not be used since it is toxic and gives false indications *in vivo* (6). Nothing has been found to date despite much search in our laboratory that will take its place in covering the zone between the ranges of phenol red and brom cresol purple,—a zone in which the reaction of many of the tissues would seem to lie.

Changes in the Coloration Result from the Injections.

In some preliminary observations, to determine the general effects of acid and alkali upon the hues of the stained tissues, relatively concentrated solutions of sodium carbonate and of hydrochloric acid were run into the blood stream.

These had the strengths employed by MacNider (7) in his studies upon kidney function, that is to say they were equimolecular with 1.5 per cent NaCl and, in the case of carbonate, 3 per cent NaCl (concentrations slightly less than N/2 and N/1); and hence they were notably hypertonic. To lessen the likelihood of anhydremia from this cause, with "outlying acidosis" as a sequel (8), several cc. of warm water was given to the animal by stomach tube some minutes prior to the injection. The acid or alkali was run in as fast as possible, which was after all rather slowly. Tests showed that about 4 cc. of N/1 Na_2CO_3 could be introduced into a rat of 170 gm. in the course of an hour, with the animal still in excellent condition, but if the rate was increased a very little, or a few drops were inadvertently run in together after the injection had proceeded for some time death occurred immediately. Since N/2 HCl greatly damages the red cells N/4 HCl was early substituted for it. More than 5 cc. of the latter solution, given during the course of half an hour, was tolerated by a rat of 140 gm. The patching characteristic of an "outlying acidosis" was never noticed.

Shortly after the administration of acid was begun the secretion of urine stopped, as in the case of MacNider's animals; whereas that of the alkali was attended by a profuse diuresis. The injections were pushed to the lethal point, but from time to time while the condition of the animal was still good, the color of bits of its vitally stained organs were compared with those from the control by the technic above described. It was soon found that there exists a group of tissues,—connective tissue, tendons, fascia, and cartilage,—which undergo marked alterations in hydrogen ion concentration, as indi-

cated by the phthaleins, when acid or alkali is given. The hue of certain other tissues, parenchymal in make-up,—those of the liver, pancreas, and lymph nodes,—remains practically unchanged so long as life lasts. The tissue which responds soonest to the alteration in the reaction of blood is the connective tissue.

Saline Solution Fails to Influence the Tissue Reaction.

More extensive experiments were now undertaken with solutions isotonic with the blood (N/6.35 solutions). Large amounts, 15 cc. or more, were tolerated by rats of 150 gm.; and there was diuresis after the acid as well as after alkali. The question arose of how far the observed alterations in the tissue colors were referable to the amount of fluid introduced, merely as such. To control this factor some injections were made of 0.92 per cent NaCl. It was found that 20 cc., run into 150 gm. rats at the rate of the acid or alkali, caused not the slightest change in the hue of the tissues stained with phenol red except the fading incident to rapid elimination of the indicator. A like result followed the injection into a 110 gm. rat of 15 cc. of isotonic dextrose solution (5.4 per cent Merck's dextrose in twice distilled water).

The findings with hypertonic solutions of acid and alkali did not differ essentially from those with the isotonic solutions save that in the case of the former the phthalein was retained longer in the body; so it will be unnecessary to discriminate between the two sets of experiments in detailing the results.

Effects of Alkali on the Coloration with Cresol Red.

The surface hue of a living rabbit stained with phenol red and viewed under oil by reflected light is indicative of a reaction between pH 7.4 and pH 7.6, as can be shown by comparing it, through an Autenrieth wedge containing water, with the hue obtained by placing on the surface of a shaved but unstained control, one or another of a series of similar wedges filled with buffer solutions of appropriate pH, stained with phenol red. The surface color of urethanized and stained rats is indicative of the same slight degree of alkalinity. The subcutaneous tissue as such appears relatively acid by transmitted light,

having the orange-pink of an apparent pH of about 7.2; and the more ruddy surface hue is to be ascribed, in part to the optical conditions and in other part to a staining of the alkaline interstitial fluids (9). When cresol red has been injected the oiled surface of the stained rat appears yellow, slightly tinged with brown, and all the stained tissues are a brilliant yellow. Several experiments were made to determine whether the color could not be altered to the rose-purple which is characteristic of a pH of 7.8 or more, under controlled conditions.

In an initial test a Na_2CO_3 solution isotonic with 1.5 per cent NaCl was gradually injected into a rat of 110 gm. The animal died when 9.0 cc. had been run in. The urine was orange-red to begin with but later purply red; yet none of the stained organs changed from the usual yellow hue. In the course of further observations, with N/1 sodium carbonate, the blood changed from orange-red toward purple, and the surface color from yellow to rose-pink, after 3.7 cc. of the solution had been run into an animal of 170 gm. stained by the injection of 1.7 cc. of 4 per cent cresol red solution. The nose, lips, and ears became light red; yet the tissues generally appeared of the same clear yellow as in the control, when the blood and intercellular fluid were rapidly pressed from them. A rat of 250 gm. given 10.4 cc. of N/6.35 sodium carbonate became ruddy orange, and its urine much more purple than that of the control. The ears *in situ* appeared ruddy and so too did a skin flap dissected up and viewed by transmitted light. But a wedge-shaped piece of the ear, rapidly excised and compressed to drive out the fluid, was as yellow as the ear of the control; and so too with all of the other tissues examined, connective tissue, cartilage, tendon, voluntary muscle, liver, lymph nodes, kidney, and pancreas. Many of these looked somewhat ruddy until pressed upon to expel the stained fluid associated with them.

It was obvious from these experiments that only the blood and intercellular fluids could be altered sufficiently in reaction by the injection of alkali to come within the range of cresol red. This would have meant a considerable change, granting,—what has not yet been proven,—that cresol red when in the body serves to indicate the pH as accurately as it does *in vitro*. For a rose-purple hue of the tissues could scarcely be expected short of pH 7.8 or 8.0; and the most alkaline of them, the connective tissue, exhibits under ordinary circumstances when stained with phenol red,—an indicator well tested in this relation (10),—the hue indicative of an approximate pH of 7.2.

Effects of Alkali on the Coloration with Phenol Red.

The color of the shaved and oiled body surface of healthy animals injected with phenol red and viewed by reflected light corresponds, as has been stated, to that associated with a pH of 7.4 to 7.6 under controlled conditions. Many of the organs, notably the parenchymal ones, are stained clear yellow, indicating that their reaction lies beyond the range of the phthalein on the acid side; but two of the matrix tissues,—the connective tissue and tendon,—exhibit shades of pinky yellow which point to the presence of a relative alkalinity within them, the connective tissue appearing to be at about pH 7.2, and the tendons at, or slightly above, pH 7.0 (11). If the introduction of alkali into the blood alters the reaction of these two tissues one would expect the change to be demonstrable with phenol red since their apparent normal reaction lies well within the range of this phthalein. Such is the actual case.

In preliminary experiments hypertonic solutions of sodium carbonate were employed. Shortly after the alkali had begun to run in the rats were noted to be purpler than the controls, and soon the general hue was a brilliant rose-purple, especially pronounced on the nose, mucous membranes, and ears. The blood coursing through the latter was seen to be intensely purple as compared with red in the controls. The urine, now voided in great quantity, was no longer stained red but purple. When the alkalosis had become pronounced examination showed that both the connective tissue and tendons had altered from pinky yellow to old rose; and the cartilage was affected as well, being now ruddy orange instead of the usual orange-yellow. Some young animals of 50 to 80 gm., in which the injection was pushed to the limit compatible with life, had flat bones of the skull that appeared pinker in the growing portions than those of the controls: but owing to difficulties of the dissection this difference cannot be stressed. The differences observed in tissues of the "core" of the tails of young animals were clear-cut, and leave no doubt that the reaction of the connective tissue, tendon, and cartilage had been altered *in vivo*. The brief ischemia incident to the examination could not have killed these hardy matrix tissues, of course. Wedges cut rapidly from the ears and pressed to force out the ruddy interstitial fluid were orange in the case of the injected animal, yellow in the control, as would follow from the fact that they consisted mainly of cartilage.

The pancreas, liver, lymph nodes, and kidney cortex were always of the same brilliant yellow as in the control even when the animal was moribund from the large amount of alkali injected. In one case the Malpighian bodies of the spleen were also observed to be yellow. The peripheral nerves were unstained but

the hue of their sheaths was changed from pinky yellow to old rose,—which one would expect from their character as connective tissue; and the walls of the large arteries were old rose. The lymph was much purpler than in the controls.

The changes observed in animals given strong alkali were duplicated when it was administered in N/6.35 solution. The isotonic carbonate solution was especially well tolerated, and we have injected 6 cc. into an animal of 65 gm. during the course of 1½ hours without causing death, and 27½ cc. into a 200 gm. animal in about the same time. Whenever there was an initial color difference in the pair of rats we regularly injected that individual which had the lighter, and sometimes the less alkaline, hue,—*jasper red* for example as compared with *eugenia red*. The changes brought about by the alkali were the more remarkable under such circumstances. As the injection progressed one saw the rat which was originally the lighter with perhaps a slightly yellow cast to the surface red, become relatively dark and purply, and those of its tissues turning toward purple appeared more markedly stained than was the case in the control, although they really had less of the phthalein in them—a fact demonstrated by applying alkali to bits of both *in vitro*. The tissues of the injected animal which did not alter from the ordinary hue, remaining yellow, were a paler yellow than those of the control because of a more rapid loss of phthalein by elimination of it from the body.

Situation of the Phthaleins.

Changes in the color of the blood can be ruled out as a cause for the alterations in the tissue hue, save in the case of especially vascular organs (ears, nose, mouth, and tongue) viewed *in situ*; for the prevailing color on staining with phenol red and other phthaleins is due to an extravascular penetration of the dye (12). It might be urged however that changes in the reaction of the interstitial fluids, secondary to those taking place in the blood, will suffice to explain the observed color phenomena. This is not the case. True, the suffusion of the matrix tissues with purply interstitial fluid tends to intensify their color. But the phthalein has a tendency to become fixed on the tissue components themselves (13). A bit of connective tissue or tendon removed from a rat stained *in vivo*, frayed with the knife, and examined under the high power, will regularly show a brilliant homogeneous staining of even its finest filaments. So too with the thinnest edge of a sliver of cartilage. The cells of such organs as stain yellow with phenol red, as if acid, are yellow even after alkali has been administered despite the presence of a purply red interstitial fluid, that is to say one which is relatively alkaline. Whether the cells of the matrix tissues as well as the ground substance become stained is not certain. It is safest certainly, for the present, to look upon the phthalein findings as expressive of the reaction within tissues, as distinct from that within cells (14).

The results obtained with phenol red were consistent. They showed not only that altering the reaction of the blood toward alkalinity

resulted in changes in the reaction of some of the tissues, but that the ones evidently affected were those of which this might have been predicted owing to the ease with which they are penetrated by substances escaping from the blood stream (15) and to the further circumstance that ordinarily their apparent reaction, as indicated by the color they assume with phenol red, lies well within the range of this phthalein. Of the tissues with reactions lying beyond the range of phenol red,—on the acid side of it,—only one was so influenced by the alkali as to alter in hue. This tissue, the cartilage, would appear to be only slightly more acid under ordinary conditions than are connective tissue and tendon, occupying in this respect a middle place between them and the parenchymal organs (16). For all that is known to the contrary the reaction of these latter organs might have been changed by the alkali without bringing them within the range of phenol red. To test for such an occurrence, as well as to obtain a check on the general findings, brom cresol purple was now employed.

Effects of Alkali on the Coloration with Brom Cresol Purple.

Brom cresol purple is not stable in a 4 per cent solution brought to pH 7.4 but undergoes a rapid chemical change (17) which leaves it something of an indicator still but with a different range and inferior color values. The deterioration can be prevented in 1 per cent solutions by a special mode of preparation (18), but at 4 per cent it begins within a few minutes at most after the titration, as a change from purple to orange, starting at one point as a rule and rapidly spreading throughout the fluid. It does not take place when there has been a slight overalkalinization, and resort was had to this procedure in order to obtain stable preparations for injection. 400 mg. of brom cresol purple was taken up in 1.6 cc. of N/1 NaOH and diluted to 10 cc. with distilled water. Had 1.48 N/1 been used instead the solution would have been at pH 7.4. The rats tolerated well the intraperitoneal injection of the phthalein treated thus, when given in the proportion of 1.6 cc. for every 150 gm. of animal. Test observations on the color of the indicator diffusing into buffer solutions from pieces of the vitally stained tissues proved that it had undergone none of the deterioration just described.

The rats stained with brom cresol purple and injected with sodium carbonate, either in isotonic or hypertonic solution (54.6 gm. to a liter, equimolecular that is to say with 3 per cent NaCl), showed striking alterations in the surface hue. They became much more purple, *dull blue-violet*, in Ridgway's nomenclature, as compared with the *dull violet-blue* of uninjected animals. The phenomenon could not be accounted for by a retention of the dye in the tissues at a time when the controls had begun to decolorize, since no such retention occurred but on the

contrary a greater elimination by the experimental animals. The alkali injection regularly resulted in profuse diuresis, with a purple urine; but the urine of the controls was purple also, that is to say alkaline, probably because of the slight excess of alkali introduced with the indicator. By injecting the control animals with sodium carbonate, at a time when they had come to appear much less purple than the experimental ones they could be brought to the same, or a deeper hue.

The blood and lymph (as pressed from the superficial lymph nodes) both appeared blue-purple prior to any administration of carbonate; and they did not become more purple after it. The fact carries implications of importance. When really at pH 7.4 to 7.5,—the reaction of rat blood (19),—brom cresol purple is so largely dissociated that further alkalinization fails to result in an increase in the purple hue appreciable to the naked eye under test-tube conditions. It follows that no change in the color of blood stained with the phthalein should occur upon alkalinization *in vivo* if the circulating indicator has still the range characterizing it under controlled conditions *in vitro*. And no change does occur either in blood or lymph. Yet there is an alteration in the color of the body surface, which becomes more purple. This can only be attributed to an alteration in the hue of the subcutaneous tissues,—the actual event as direct inspection shows. These tissues, less alkaline than the blood, as judged by their hue with phenol red, must be less alkaline too with brom cresol purple; for otherwise the injection of sodium carbonate would not cause their color to intensify. The results with the two indicators are consistent in the demonstration of this difference in relative reaction.

Not only does the connective tissue of the animal injected with carbonate become more purple but so too do the tendons and cartilage. When a segment of the ear, which consists mainly of the last of the tissues mentioned, is excised and compressed between mica surfaces it can be seen to be far more purple than the control segment; whereas the almost blood-free fluid extruded from it and protected by oil has the same hue in both cases. The core of the tip of the tail of the injected rat also shows a more purple cartilage, and the costochondral articulations are a deeper purple. The bones stain poorly, and no satisfactory color comparison is to be had with them. In contrast to the ease with which the hue of most of the matrix tissues is altered that of the parenchymal organs evinces no change. The blue-green or yellow-green color of the lymph nodes, liver, and pancreas remains the same even when so much alkali had been introduced that the animal is in a moribund condition.

Valentine knife sections of the kidney cortex of our alkalinized animals were purple to the eye as compared with the yellow-green of the control tissues; and under the microscope one could see that the tubules contained a far more intensely purple urine and that a great many of them were colored purply blue. The differences will not be gone into however since they were the product of especially complicated conditions. An account of them will be reserved for a later paper from this laboratory.

These observations concluded the series on the effects of injected alkali on the coloration of tissues stained with phthaleins. The results of the various experiments were in complete accord in showing that there exists a group of tissues—matrix tissues—which become more alkaline when the blood is alkalinized; and a second group of tissues, parenchymal in constitution, which are to all appearance unaffected even when alkalinization is pushed to the extreme. Tests were now begun upon the effects of acid.

Effects of Acid on the Coloration with Phenol Red.

There are few tissues staining with phenol red of which any color alteration can be expected on the injection of acid; for only a few have internal conditions bringing them within the range of this indicator, the reaction of the others appearing to lie beyond it, on the acid side (20). The connective tissue and tendons stain yellow-pink and pinky yellow respectively; and these colors might conceivably be altered to a frank orange-yellow when acid is administered. This has proved to be the case.

When N/6.35 HCl was gradually run into a jugular vein of the stained rat the body surface, which had been *jasper red*, became *coral red*, then *carnelian red* and eventually *rufous*. Meantime the connective tissue turned from salmon to orange-yellow, and the tendon, which had not been so pink to begin with, took on the same hue. The blood and lymph became somewhat less rosy than usual. The other tissues, being ordinarily orange-yellow, did not alter in hue. There was some diuresis, of acid urine stained with phthalein, but the changes noted in the color of the tissues, cannot be ascribed to an unusually great elimination of dye: for the application of alkali to specimens from the control and experimental animal rendered them equally and deeply rose-purple.

Effects of Acid on the Coloration with Brom Cresol Purple.

An extensive series of observations were carried out with brom cresol purple. The blood and lymph did not alter notably in hue as acidosis developed, yet there were great changes in the surface color, the hue turning from the *dull violet-blue* of Ridgway to *grayish violet-blue*, or, if the animal was more deeply stained to begin with, from *deep soft blue-violet* to *grayish blue-violet*. The alterations were mainly the outcome, as further findings showed, of changes in the hue of the connective tissue.

Skin flaps dissected up in such wise as not to disturb the circulation markedly, appeared pale greenish blue in contrast with the intense purply blue of the control, when viewed under oil by transmitted light. The ears were purply green, not a frank purple as in the uninjected animal; and when segments were cut and rapidly compressed between mica slides to drive out fluid, the one appeared brilliant green and the other a frank purple. The extruded fluid, protected by oil, was in both cases purply blue. No better evidence of the independence of the tissue color could have been desired. Valentine knife sections of the ear of the experimental animal showed a blue-green, or even yellow-green, connective tissue, as compared with the deep blue of the control, and a light green, or greenish blue, cartilage, that of the control being a deep purply blue. The tendons, purply blue in the latter animal, were a paler blue or else green in the rat given acid, and a like contrast was to be observed in the wall of the femoral artery. These differences cannot be laid to a greater decolorization as result of the injection; for the application of alkali to pieces of the tissue showed that those from the injected animal contained as much and often more of the phthalein.

The color of the parenchymal organs appeared practically uninfluenced by the acid, save in the case of the kidney. The renal cortex, as viewed in the gross, had undergone an obvious change toward yellow, though the microscope showed much of the difference to be due to a more acid secretion, the fluid within the tubules being green, not purple as in the control. There was, in addition, some yellowing of the tubular epithelium. The pancreas, though, and the lymph nodes, never altered from the ordinary yellow-green staining, nor did the liver from its characteristic blue-green except in animals moribund from the acidosis, when it was noted to have changed very slightly toward yellow. The change may conceivably have been due to alterations in the reaction of the bile which was deeply stained (21).

It will be seen that the findings corroborated those with alkali in pointing to the existence of a group of tissues readily influenced in reaction by the introduction of acid or alkali into the blood stream, and of another group not susceptible to such change. And, as in the experiments with alkali, brom cresol purple gave information confirming and extending that obtained with phenol red.

The Changes Resulting from Injections of Lactic Acid.

Some subsidiary tests were made on the effects of lactic acid in isotonic solution (0.314 N) upon animals stained with phenol red. The injection fluid was tolerated in large amounts, 14.5 cc. failing to cause the death of a rat of 126 gm. into which it was run in the course of only a little over an hour; and unless a flow into the jugular was

continuously kept up not even a change in the surface color took place. By hurrying the injection a surface change from *jasper red* to a hue between *carnelian red* and *vinaceous-rufous* could be brought about, and under such circumstances alterations were to be noted in the color of the connective tissue, cartilage, and tendons, resembling those when hydrochloric acid had been used. But as soon as the injection was intermitted the surface color began to return toward the normal, as doubtless did the hue of the tissues mentioned, though observations were not made upon the point.

DISCUSSION.

The foregoing experiments make clear the fact that when acidosis or alkalosis is induced by the gradual injection of hydrochloric acid or of sodium carbonate into the blood stream changes take place in the reaction of some of the tissues. We have not sought to determine how great an amount of acid or alkali is required to cause these changes, nor have we made detailed observations upon the influence of the rate of injection. In most instances enough of the solution to cause death was eventually run in, but the examination was made while the animal was still in good condition save in the case of those tissues which the preliminary work had shown to be resistant to change. The solution could be allowed to enter rapidly at first, because of the buffering action of the blood, but later only drop by drop, so timed that each drop took some seconds or minutes to flow in. At first, too, because of the buffering action just mentioned, no change in the color of the tissues occurred. A few drops of the solution given late in the injection period often had more effect than did as many cc. earlier.

The question may be asked whether the changes were not the result of gross injury to the walls of the blood vessels with effusion of the acid or alkali. This possibility can be ruled out at once. The animal organism will tolerate but slight alterations in the reaction of the blood; and the color phenomena elicited by the injection of weak solutions (N/6.35) were the same as with strong ones (N/2 N/1). Further, the acid and alkali were of a kind to yield innocuous salts. It is true that even N/6.35 HCl acts to transform into acid hematin the hemoglobin coming directly into contact with it. But the damage

to the blood produced in this way under the circumstances of the experiments was not of itself sufficient to explain the issue of them. In a later paper some figures will be given on the anemia produced in rabbits by the intravenous injection of the maximum quantity of hydrochloric acid compatible with life. The blood loss is surprisingly slight.

How now are the changes in the relative reaction of the tissues to be accounted for? By changes in the ability of the blood to carry oxygen and carbon dioxide? This factor may have importance in the case of the animals given acid, owing to the effect of the latter to lessen the affinity of hemoglobin for oxygen, and thus to reduce the effective concentration of the gas. Asphyxia of the tissues is a well known cause of acid formation within them; and toward the end of some of our experiments the animals manifested air hunger. Acid lessens as well the ability of the blood to transport carbon dioxide; and the presence of this gas in the tissues of the stained, acidotic animal can be sufficiently shown by laying them open, when they rapidly become more alkaline, as evidenced by a change in hue. But this happens in the case of the similarly ventilated tissues of controls as well (22). When one alters the reaction of the blood in the direction of acidity one alters also the gradient responsible normally not alone for the passage of carbon dioxide from the tissues into the blood but for that of the acid products of metabolism generally. Here is yet another possible reason for the acidosis. This may have been due in no inconsiderable part to a damming back of substances which would normally escape to the circulation and be excreted, instead of to an extravascular penetration of hydrogen ions introduced with the injection fluid.

May one suppose that under the circumstances of blood alkalosis the gradient just referred to becomes so steep that the tissues are stripped, so to speak, of carbon dioxide and other acid products as soon as these are formed, with result that the local reaction becomes more alkaline than the normal? Such an occurrence is conceivable, since stained normal tissues ventilated by exposure to air soon become as alkaline as they ever appear to be after an injection of alkali.

The tissues which alter in reaction when alkali or acid is run into the organism,—connective tissue, cartilage, and tendon,—are those

characterized by the presence of matrix substance in large quantity, a matrix which stains deeply with the phthaleins. As pointed out in previous papers these tissues appear to be largely passive in behavior. They control their own conditions only where the influence of the separated cells is immediately effective; and when stained with phthaleins and immersed in weak buffer solutions they behave almost like so much indicator paper (23). Their metabolic rate is very low as compared with that of the parenchymatous organs. One may ask whether the intercellular substances which constitute so large a part of their bulk are alive at all in any proper sense of the word. It is easy to suppose that the changes in the reaction of these tissues are the direct result of an exchange of ions between them and the altered blood, without invoking other causes. But the precise interpretation of our findings cannot now be attempted for lack of data.

To give reasons for the failure of the parenchymatous organs to undergo changes in reaction is another, and more difficult matter. True, the cells of these organs may be thought of as making their own conditions (24), one of these being under ordinary circumstances,—or so it would appear,—a not inconsiderable acidity. But there is no evident increase in this local acidity in animals rendered acidotic, despite the great metabolic activity of the cells; and no diminution in it even when alkalosis is pushed to the extreme. It is possible, not to say certain, that the abilities of cells to maintain a normal state of affairs within their cytoplasm are often greater than those for survival of the body as a whole. Yet quite possibly too the phthalein of our experiments may in the parenchymatous organs have become so associated with some of the cell components that it no longer served as an indicator. All of the observations made in this laboratory prior to the ones here reported are against this explanation, however.

The changes toward acidosis or alkalosis sustained by the organism under the conditions of nature differ from those we have reported in that they are not ordinarily caused by a direct introduction into the blood of H or OH ions from without, though often referable to an indirect passage of them into the circulation by way of the gut. A large proportion of clinical instances of alkalosis or acidosis derive from some upset here or there amongst the organs. But except in the immediate region of the upset the net result must be the same in the

end in its effects upon the tissue reaction as if the disturbance had been a general one. That in pronounced cases an alteration takes place in the reaction of the matrix tissues can scarcely be doubted.

There is much on record to show that the introduction of acid into the body leads to a withdrawal of base from the tissues; and the quantity lost may well alter the composition of these latter. One wonders whether the matrix tissues, which are so readily permeable from the blood, may not serve as a second line of buffers, acting as interstitial tissues, tissues that fill up cracks, in a physiological as well as an anatomical sense. It would be in keeping with what is termed the body economy if a material like cartilage should prove useful in other ways than by keeping the ears at an angle from the head and the joints of the limbs well rounded.

Somewhere in the literature surely, there must be indirect observations upon the participation of the tissues in the buffering activities so beautifully illustrated by the blood; for the extent of the tissue participation can be come at, since the total blood value as a buffer is calculable and the changes incident to excretion can be controlled. The only observations we have been able to find, however, are those of Atzler and Lehmann (25) who perfused the isolated hind legs of dogs with acid and alkaline solutions over long periods of time and came to the conclusion that the greater the initial degree of acidity or alkalinity the greater was the change toward neutrality undergone by the perfusate.

Our observations by the indicator method upon the changes occurring in the tissues when acid or alkali has been run into the blood stream are not the first upon this theme. Henning (26) injected HCl or NaOH into mice and sought to determine the reaction of the organs after the method of Gräff (27) by dipping pea-sized pieces of them into drops of indicator solution placed on a glass slide. The animals had been decapitated, or had died as direct result of the injection; and the specimens stained after death contained the blood into which acid or alkali had been run. Under these conditions Henning noted that when a great deal of acid or alkali had been placed in circulation practically all of the tissue pieces except those from the brain appeared to contain H or OH ions in abnormal quantity.

Some of the rats of the present work withstood so well the introduction of N/6.35 HCl and the resulting marked changes in tissue reaction that we have been led to make observations upon the course of tissue acidosis as thus induced. The work constitutes an elaboration in certain directions of that here reported. It will be the subject of a succeeding report.

SUMMARY.

The introduction into the blood stream of dilute hydrochloric acid or sodium carbonate in quantities not too great to be compatible with life results in marked alterations in the color of certain of the matrix tissues stainable *in vivo* with phthalein indicators. Connective tissue in its various forms, and tendon and cartilage all become relatively more acid or alkaline than the normal. The hue of the kidney cortex also changes, as might be expected from its functions. The pancreas and lymph nodes, on the other hand, appear unaffected even when the injection is pushed to the extreme; and the slight to negligible alterations in the hue of the liver may be due to changes in the color of the associated secretion. The matrix tissues just mentioned behave as if unable to maintain a reaction of their own; whereas the elements of the parenchymal organs would seem to make their own conditions even when so much acid or alkali has been injected as will lead to death of the animal.

Injections of lactic acid are well tolerated and it is difficult to bring about alterations in the color of the phthalein-stained matrix tissues by means of them. Salt solution, and sugar in large amount cause no changes in color such as would indicate changes in reaction.

The results with the various phthalein indicators are in close accord, attesting that the information these give under vital conditions is reliable.

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A FILTERABLE VIRUS PRESENT IN THE SUBMAXILLARY GLANDS OF GUINEA PIGS.

By RUFUS COLE, M.D., and ANN G. KUTTNER, Ph.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

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During recent years much attention has been paid to peculiar alterations in the morphology of certain cells in lesions associated with the presence of filterable viruses. These changes are especially constant and well marked in the lesions of spontaneous and experimental herpes simplex. Indeed, they occur so constantly in this condition that Goodpasture and Teague (1) have used the presence of these cells as a guide in tracing the passage of the virus through the tissues of the infected animal. The uniformity of the cellular changes in herpes simplex and the constancy with which they occur renders this the most satisfactory of the virus diseases to be used as an example in discussing analogous and related conditions. Cells derived from the endothelium, epithelium, or mesenchyme may show these changes and although there may be variations in the appearance of the alterations in the different cells, on the whole they are very similar, whether the cells involved be connective tissue cells, epithelial cells of the cornea, or large ganglion cells of the central nervous system.

The characteristic features of these changes, as seen in tissues stained with eosin and methylene blue, are the following. The nucleus takes on a vesicular character and the limiting membrane is deeply stained with the basic dyes. Usually the inner surface of the membrane is irregular, as though the basic staining material were collected in granules or small clumps. A few of these granules are much larger than the others and these are considered to represent the nucleoli of the unaltered nucleus. Within the nucleus, usually at the center, is a round or oval body which stains either faintly or, more often,

deeply with acid dyes. The depth of staining depends to some degree, however, upon the length of exposure. The red-staining material may occupy not more than half, or even less, of the nuclear space or it may occupy almost the entire nucleus. In any case it is sharply limited from the surrounding nuclear material by a clear unstained halo. The material itself is usually granular, never hyaline or dense; it appears as though composed of a multitude of very fine granules compressed into a ball or mass.

Nuclear changes which cannot at present be differentiated from those occurring in the lesions of spontaneous and experimental herpes simplex also occur in the skin lesions of herpes zoster, in the skin lesions of varicella, and in the lesions experimentally produced in rabbits by the Virus III of Rivers and Tillett (2). Nuclear changes resembling to some extent those occurring in herpes simplex are also found in a variety of conditions, especially diseases of animals, such as, epidemic encephalomyelitis of horses, or Borna's disease, fowl-pox, certain diseases of fishes, etc.

Except for a few reported instances, nuclear changes like those occurring in herpes simplex have so far been found only in conditions in which the presence of a filterable virus has been demonstrated or in which the association of a virus of this group is very probable. The experimental production of these lesions except by the injection of filterable viruses has, in our hands, been unsuccessful. It is true that Luger and Lauda (4) have mentioned the occurrence of similar structures in a case of salvarsan dermatitis. But even though these lesions should be present in isolated instances of this kind, it would be necessary to demonstrate the absence of a filterable virus in the given instance before the present conception of the direct relationship between these nuclear changes and filterable viruses would become untenable.

Lipschütz (3) has considered that these nuclear changes represent "nuclear inclusion bodies" in the sense of Prowazek. He has collected all the conditions in which nuclear inclusions occur, and also the conditions in which unusual bodies or structures are found in the cytoplasm of the cells, into a great group of virus diseases, the causative agent of which he classes with the *Chlamydozoa-strongyloplasma*. Lipschütz has maintained that the bodies or structures seen within the nucleus represent a specific reaction of the cells to a living virus.

The bodies are not considered to be masses of parasites but are held to represent reaction products, associated with which is the virus.

This conception of Lipschütz has not been universally accepted, however. Luger and Lauda (4) who have devoted much study to the nature of these nuclear changes, maintain that they do not represent "inclusion bodies" in the sense of Prowazek, but that they are the result of a non-specific type of nuclear degeneration, which these authors call "oxychromatic degeneration." They refer to the observations of Heidenhain, who described two varieties of nuclear, chromatic substance, basic chromatin and oxychromatin. During nuclear degeneration occurring under certain conditions, the oxychromatin tends to collect in the center of the nucleus and the basic chromatin at the periphery. According to these writers the typical nuclear changes seen in the lesions of herpes simplex represent the final stages in this separation of oxy- and basic chromatin.

At the present time the evidence in favor of either of these views is not convincing. Our own observations suggest that the inclusion bodies are produced by the accumulation of a finely granular material which at first is scattered throughout the nucleus. This material which, in its scattered form, is very faintly acidophilic, takes on a deeper and deeper red stain as it accumulates into a mass, until the typical nuclear changes become manifest. The earlier stages of this process, however, are difficult to detect and usually only the fully developed nuclear "inclusion body" is seen. Until further knowledge concerning the chemical nature of these structures is obtained, it is not important, at least in the present connection, to decide whether we shall speak of "nuclear inclusions" or of "oxychromatic degeneration" of the nucleus. It is very important, however, to know whether these changes represent characteristic lesions due to the action of filterable viruses or whether they represent a form of degeneration which occurs under a great variety of conditions producing a non-specific injury to the cell. The burden of evidence at present points to the former concept, for it would be very surprising, in view of the careful study which has been made of cellular changes under various pathological conditions, that this striking nuclear alteration should have been frequently overlooked.

In a few isolated instances, pathologists have reported finding peculiar nuclear changes in human pathological material. VonGlahn and Pappenheimer (5) have recently collected the reports of sixteen human cases, and have added a case of their own, in which, at autopsy, in various viscera, cells with striking nuclear abnormalities were present. While the descriptions of these nuclear changes in the individual cases differ to some extent, in most instances they are sufficiently alike to justify the conclusion that the various writers were dealing with identical or closely related conditions. All of the cases reported, except that of VonGlahn and Pappenheimer, were in infants under 1 year, five of these were still-born. Five of them probably suffered from congenital syphilis, in one nephritis was present, in one pneumonia, one had "green stools," edema of feet, and bronchitis, another one hydrocephalus and focal interstitial nephritis. The case of VonGlahn and Pappenheimer was that of a male aged 36 years, who suffered from an abscess of the liver. Certain cells in the intestines, liver, and lungs were found to be of very large size, measuring at times 25 micra in diameter. Some of these were multinucleated. Within the nuclei of these cells were large acidophilic masses. In the lung these large cells were usually in continuity with the epithelium, but cells of granulation tissue and of the blood vessels also contained the nuclear inclusion masses.

In addition to these cases collected by VonGlahn and Pappenheimer, L. Jackson (6) has reported finding an ameba-like organism in the kidneys of a child. This child, 15 months old, suffered from diphtheria and died suddenly. At autopsy lesions of bronchopneumonia were found. In the kidney tubules were large cells which the writer interpreted as amebæ. It seems from the description that these may have been structures similar to those present in the cases collected by VonGlahn and Pappenheimer. Indeed, Jackson stated that the structures were similar to those described by Ribbert (7) and by Smith and Weidman (8), whose cases were included by VonGlahn and Pappenheimer in their series.

In most of the earlier human cases the nuclear inclusions were thought to be due to the presence of parasites, amebæ, or sporozoa, in the later cases they were described as nuclear degenerations. One of the cases in infants was reported by Goodpasture and Talbot (9) in 1921. These writers drew attention to the similarity between the nuclear changes in this case and the nuclear changes in the epithelial cells in varicella which were first described in 1906 by Tyzzer (10). They also called attention to peculiar cellular changes present in the epithelial cells of the salivary glands of guinea pigs which were first described by L. Jackson in 1920. Goodpasture and Talbot studied these cells in guinea pigs and concluded that the condition was an example of cellular transformation similar to that occurring in the lesions of infancy. They thought that there was no evidence that these cellular and nuclear changes were due to some intracellular infection. Von Glahn and Pappenheimer were the first to fully identify the large cells occurring in human cases with the abnormal cells met with in herpes simplex and related conditions. They regarded the intranuclear masses seen in their own case as nuclear inclusions, "identical in their morphology and staining reactions with the

bodies seen by previous observers in the viscera of infants, and by Lipschütz and others in tissues of spontaneous and experimental herpes, and in various neural and visceral lesions produced by the herpetic and related viruses." These writers were unable to carry on any experimental study, but offer the suggestion that the lesions in this case may have been related to the presence of an unknown virus.

Cellular Changes in the Submaxillary Glands of Guinea Pigs.

Unusual changes in the submaxillary glands of guinea pigs were first described by L. Jackson (11) under the title, "An intracellular protozoan parasite of the ducts of the salivary glands of the guinea pig." These structures were found in 26 of 48 pigs examined. She interpreted them as protozoa, probably coccidia. We have had no difficulty in confirming the observation of Jackson that in the ducts of the submaxillary glands of guinea pigs there are found unusual and striking structures which on first observation suggest a parasitic origin. However, we have been unable to differentiate the different stages of development resembling those of protozoa which Jackson described. The structures we have observed, however, conform in all particulars with those shown in the illustrations in Jackson's paper and there can be no doubt that we are dealing with the same abnormalities. We have examined the submaxillary glands of 75 guinea pigs over 6 months of age. Sections from these glands have been stained in eosin and methylene blue, and in 63 of the glands, or 84 per cent, these unusual structures have been found in larger or smaller numbers. From our own study we have identified them as swollen epithelial cells (Fig. 1). The nucleus of each of these cells contains a mass of granular material which is definitely acidophilic. The altered cells are found chiefly in the ducts of the serous portion of the gland, though, in a few instances, they have been seen in the mucous portion. The cells lie either on the basal membrane contiguous to the unaltered epithelial cells or within the lumen of the duct, evidently having pushed forward during the process of hypertrophy. The altered cells occasionally are not more than twice the usual size (Fig. 2) but in most instances they are much larger than this, up to 40 micra in diameter. The large size and red staining of the nuclear inclusions render them easily visible under the low power of the microscope. In ducts cut obliquely or longitudinally, not infrequently six or eight, or even more,

of these structures can be seen within a single duct; in other ducts not more than one or two of these cells are present. In the sections from certain glands only one or two groups of ducts containing the altered cells are seen, in other glands large numbers of ducts are involved. About the ducts containing these cells there is usually a cellular reaction and this is often of assistance in locating the areas in which the altered cells are present. The tissue reaction about the involved ducts consists of mononuclear cells, lymphocytes, and large cells with vesicular nuclei (Fig. 3). Polymorphonuclear leucocytes are rarely seen and never predominate. The cytoplasm of the involved cells usually takes on a light blue color which, however, is considerably more intense than that of the cytoplasm of the unaltered cells. The nuclear membrane is somewhat irregular, and stained deeply with the basic dye. At or near the center of the nucleus, and separated from the membrane by a clear unstained area or halo, is a mass which is stained red. The color of this mass is usually definitely deeper than that of the typical herpes simplex inclusion bodies. This mass may occupy not more than one-fourth the nuclear space but it not infrequently fills almost the entire space, leaving a very narrow halo between it and the membrane. Within this clear space there are seen two, three, or even more, irregular masses, 0.5 to 2 micra in diameter. These masses stain deeply with the basic dye. They may lie approximated to the inner surface of the membrane, or upon the acidophilic mass, or free in the space. Upon careful focussing in almost all instances radiating irregular lines passing from the mass to the inner surface of the membrane can be observed. These bands also stain with the basic stain. Occasionally a very faint network of strands between the various radiating bands is apparent. The central mass is usually oval or round, the margin is slightly irregular, and the mass appears finely granular. At times some of the granules appear larger and somewhat refractile. The presence of these granules may have been responsible for the interpretation of these structures as protozoa and for the descriptions of an organized structure. It is evident from this discussion that in their main features the cells resemble those occurring in the lesions of herpes simplex. On account of their much greater size and the darker color of the inclusion bodies the resemblance is, at first, not evident.

Sections have been made from the submaxillary glands of a number

of animals of other species to determine whether identical or analogous structures are also present in them. The glands of eight full grown rabbits, three rats, a mouse, a dog, and a cat, have been studied but no structures resembling the large cells seen in guinea pigs have been found. Although these peculiar cells were present in the submaxillary glands of 84 per cent of the guinea pigs over 6 months old, in very young pigs they were present only occasionally. Of 43 young guinea pigs, most of them less than 1 month old, the large cells were found in the submaxillary glands in only three instances and then they were few in number and confined to one or two groups of ducts. It seems, therefore, that if these lesions are due to an infection with a virus, the infection usually occurs only after the first few weeks of life but finally almost all guinea pigs become infected.

EXPERIMENTAL.

Transmission of Infection to Young Guinea Pigs.—An attempt was first made to demonstrate whether or not an infectious process is responsible for these lesions. The most obvious mode of procedure would be the inoculation of material from glands in which the lesions were presumably present into other glands which were not already the seat of the lesion. Since, however, the lesions are found in almost all older guinea pigs it would be necessary to inject the material into the glands of very young guinea pigs free from infection. Injections into the submaxillary glands of very young guinea pigs, however, are not without difficulties, and moreover, there is always the chance that even the young guinea pigs are already infected. Nevertheless, a number of experiments have been made in which material from the glands of old guinea pigs was injected into the glands of young ones. The pigs were killed at varying periods, from 2 to 12 days, following the injection and sections were made from the submaxillary glands. In nine out of eleven injected glands a marked mononuclear reaction involving the interstitial tissue was present. In certain areas cells were found which showed nuclear changes, with acidophilic inclusions, exactly resembling those seen in the lesions of herpes simplex. Occasionally similar nuclear changes were seen in duct cells of normal size. In a very few instances the duct cells showing these changes were

somewhat swollen but they did not equal in size the very large cells in the glands of full grown guinea pigs. Most of the transmission experiments, however, have been made by injecting the material in other locations in young pigs where these cells do not occur, such as the testicle, brain, lung, and tongue. The most striking results were obtained when the injections were made into the brain and in most of the experiments this has been the site of inoculation.

Minor modifications of the technique of the brain injections were made in certain experiments and the results were not always identical but the following description is typical of this experimental method and of the results obtained. A full grown guinea pig is killed and the submaxillary glands removed under sterile precautions. A small piece is removed from each gland and placed in Zenker's solution, to be later examined in order to make certain that typical lesions are present. Aerobic and anaerobic cultures of the submaxillary gland are made at this time to exclude the possibility of bacterial infection. The remainder of the glands is cut into small pieces with scissors and ground in a mortar in about 2 cc. of Locke's solution. This is then centrifuged at low speed for a few minutes, to remove the large particles, and the supernatant fluid is used for injection. 0.1 cc. of this suspension is then injected directly into the brain[1] of a guinea pig less than 1 month old. 24 hours following this injection the temperature of the injected pig is usually not elevated and the animal appears normal. After 48 hours, however, the temperature frequently becomes elevated, 105–106°, but without the animal showing any marked symptoms. On the 3rd day the guinea pig appears sick, the hair is raised, the animal fails to move about in the cage, and the temperature continues elevated. On the 4th day, the symptoms have become more marked. The animal now begins to show signs of heightened nervous irritability as indicated by tremors and slight convulsive movements. On the 5th day it is usually very ill, has irregular jerking movements, is unable to rise when placed on its side, and death ensues. When the brain is removed no gross abnormalities beyond congestion are seen. Cultures are made to rule out the possibility of bacterial infection, and the brain is placed in Zenker's solution.

[1] Guinea pigs were anesthetized with ether before injection.

Later when microscopic sections are examined there is found well marked exudate over the surface of the entire brain, including the cerebellum (Fig. 4). The exudate consists chiefly of mononuclear cells, lymphocytes, and large cells with vesicular nuclei. There is considerable edema and the blood vessels of the membranes are distended and filled with blood. In contrast with the lesions resulting from intracerebral infection of the brain with herpes simplex virus, very slight if any changes can be detected in the brain itself, the blood vessels of which appear normal. The most striking feature is the presence in the meningeal exudate of large numbers of cells, each of which contains an acidophilic mass within the nucleus. These cells resemble in all particulars the cells containing nuclear inclusion bodies which occur in herpes simplex and related conditions (Fig. 5). The number of abnormal cells present in the guinea pig lesion is much greater than the number usually present in the lesions following injection of herpes simplex virus or Virus III of Rivers. In no case have very large cells like those found in the submaxillary gland been found. 54 young guinea pigs have received intracerebral inoculations of an emulsion of the submaxillary gland of full grown guinea pigs. Most of these animals showed symptoms similar to those described, though in some the symptoms were delayed. The animals died or were killed at various times from the 2nd to the 12th day following the injection. In 48 of the guinea pigs, or 89 per cent of those injected, lesions as described above were found.

Emulsions of the submaxillary gland of full grown guinea pigs have also been injected into the testicles of sixteen young guinea pigs. In all cases a histologic examination of the submaxillary gland was made to make certain that the specific lesion was present in the material used for injection. 0.1 cc. of the emulsion was usually injected into each testicle. The inoculated guinea pigs showed some elevation of temperature for several days, but they developed no other symptoms. The animals were killed at various intervals from 4 to 10 days following the injection, and both testicles were removed. Histologic examination was later made of one testicle from each animal and in almost all cases some degree of cellular infiltration was found. In twelve instances, or in 75 per cent of the animals inoculated, cells containing typical nuclear inclusion bodies were present. These

were either in cells of the interstitial tissue, or in cells of the tubules, or in both.

Injections of an emulsion of the submaxillary glands of full grown guinea pigs were also made into the tongues of young guinea pigs. These animals developed no symptoms. They were killed and the tongues were removed on the 3rd, 5th, and 9th days following the injection. Microscopic study of these tongues showed a localized cellular reaction in the stroma, and cells containing typical nuclear inclusion bodies were present. A tongue removed on the 9th day after inoculation showed the most marked cellular infiltration. In no instance was the epithelium of the tongue involved.

Inoculations were also made into the lungs of three guinea pigs. One of these animals showed a rise of temperature on the 7th day, and was killed. The lung showed no gross changes but on microscopic examination a circumscribed mononuclear cellular infiltration was found. In a few of the alveoli in the involved area, large mononuclear cells containing typical nuclear inclusion bodies were present. Two other guinea pigs inoculated in the lungs were killed on the 11th and 22nd days after injection. Although a mononuclear cell infiltration was present in the lungs, no cells with nuclear inclusion bodies were found. The injection of emulsions of submaxillary glands of full grown guinea pigs into young guinea pigs therefore has quite regularly resulted in the production of a subacute inflammatory reaction and the appearance of cells containing nuclear inclusion bodies morphologically identical with those seen in herpes simplex and allied conditions.

Control Experiments.—These experiments suggest that the submaxillary glands of full grown guinea pigs contain a virus which is responsible for the reaction in the young guinea pigs, and it seems probable that the large cells and the reaction seen in the older pigs is a manifestation of natural infection with this virus. This view is supported by the following observations. Four young guinea pigs were inoculated in the brain with an emulsion of the submaxillary glands of other young guinea pigs, presumably tissue containing no large cells with nuclear inclusions. Subsequent microscopic examinations showed that there were actually no lesions in these glands. The results of these inoculations were entirely negative. Nevertheless, it seemed possible that the reaction observed in the tissues of young guinea pigs

following the injections of the glands of old guinea pigs was of a non-specific nature, and that any glands of external secretion might contain irritating substances which on injection into the brains of young guinea pigs might give rise to an inflammatory reaction, and that under these conditions cells containing nuclear inclusions might appear. Consequently, two young guinea pigs were injected with emulsions made from the pancreas of an old guinea pig and seven young guinea pigs were inoculated with emulsions made from the submaxillary glands of full grown rabbits. No symptoms resulted from any of these inoculations and the microscopic examination of the brains failed to reveal any lesions. Moreover, nine young rabbits, inoculated intracerebrally with an emulsion made from the submaxillary glands of full grown rabbits, remained well and no lesions were found in the brain. The results so far reported, therefore, indicate that an infectious agent is probably responsible for the lesions found in the submaxillary glands of full grown guinea pigs and that the lesions in young guinea pigs resulting from the inoculation of the emulsion of submaxillary glands of full grown guinea pigs are due to this infectious agent.

Transmission in Series.—This conclusion would only be justifiable, however, if it were possible to reproduce the lesion after passage through a series of animals. But in spite of the fact that young guinea pigs which have received intracerebral inoculations of emulsion of submaxillary glands of old guinea pigs almost invariably show severe symptoms with marked cerebral lesions, the inoculation of emulsions of the brains of these experimentally infected animals into other young guinea pigs has invariably failed to cause symptoms or to give rise to lesions. But even though the second animal has shown no lesions or symptoms the inoculations have been continued from animal to animal in series through as many as six guinea pigs with the hope that in this way the virus might gradually acquire greater virulence. But the results have all been negative. Attempts were also made to transmit the virus by inoculating from testicle to testicle or from salivary gland to salivary gland. Except in one instance, to be mentioned later under Series C, when a testicle to testicle inoculation gave a mildly successful result, these experiments were also unsuccessful. Recourse was then had to the expedient of varying

the seat of inoculation, inoculating first in the brain and then in the testicle or *vice versa*. A large number of these experiments have been carried out and in seven instances has it been possible to obtain a positive result on the second transfer. In three of these experiments the transfer was made from brain to testicle, in three others from testicle to brain, and in one from testicle to testicle. In one series it has been possible to reproduce the lesions through a series of three transfers (Series T). In this instance the transfer was made through testicle, brain, testicle. The following are abstracts of the protocols of the experiments in which positive results were obtained.

Series M—Brain to Testicle.—March 1, 1926, Guinea Pig 1 received an injection into the brain of 0.1 cc. of an emulsion of the submaxillary glands of two full grown guinea pigs. In this animal the temperature was elevated from the 3rd to 7th days following the injection, reaching 105.8°. The guinea pig showed marked symptoms, became prostrated, and was killed on the 8th day. Sections from the brain of this animal showed a marked meningeal exudate, and typical nuclear inclusions were found in many of the cells. The remainder of the brain was emulsified and 0.1 cc. of the emulsion was injected into each of the testicles of Guinea Pig 2. In this animal the temperature was elevated to 104.4–105° from the 3rd to the 11th days. It was killed on the 11th day. Sections from the left testicle showed a mononuclear infiltration and a few cells contained typical nuclear inclusions.

Series N—Brain to Testicle.—March 26, 1926, Guinea Pig 3 received an intracerebral injection of 0.1 cc. of an emulsion made from the submaxillary glands of two full grown guinea pigs. The animal became very sick on the 3rd day and was killed. Sections from the brain showed a marked meningeal infiltration with numerous cells showing typical nuclear inclusions. The remainder of the brain was emulsified and 0.1 cc. of the emulsion was inoculated into each of the testicles of Guinea Pig 4. The animal showed an elevation of temperature from the 7th to the 9th days, ranging from 104.4–105.2° and was killed on the 9th day. Sections from the testicle showed a slight cellular reaction and in a few cells typical nuclear inclusions were found.

Series P—Brain to Testicle.—March 18, 1926, Guinea Pig 5 received an intracerebral injection of 0.1 cc. of an emulsion of the submaxillary glands of two full grown guinea pigs. The animal showed an elevation of temperature and marked symptoms, and died on the 4th day. An emulsion made from the brain of this animal was injected into both testicles of two young guinea pigs, Nos. 6 and 7. On the 7th day Guinea Pig 7 showed a temperature of 105.4° and was killed. In sections of the testicles, however, no lesions were found. Guinea Pig 6 had a temperature ranging from 104.9–105.6° on the 7th to 9th days. The animal was killed on the 9th day and in sections of the testicle a circumscribed mononuclear

reaction was seen and in the cells of the tubules in this area there was found a small number of cells, in the nuclei of which were typical acidophilic masses.

Series C—Testicle-Brain: Testicle-Testicle.—January 5, 1926, Guinea Pig 8 received an injection into the left testicle of 0.1 cc. of an emulsion made from the submaxillary gland of an adult guinea pig. This animal had an elevated temperature from 104.8–105.7° on the 6th, 7th, and 8th days, and it was killed on the 8th. Sections made from the testicle showed a marked infiltration with mononuclear cells, and in a small number of the tubule cells, typical nuclear inclusions were found. The remaining portion of the testicle was emulsified in Locke's solution and 0.1 cc. was injected into the brain of Guinea Pig 9 and into the left testicle of Guinea Pig 10. Guinea Pig 9 showed an elevation of temperature on the 5th to the 9th days, ranging from 104.7–105.8°. On the 9th day this animal was killed and sections made from the brain showed a circumscribed meningitic exudate in which were numerous cells showing typical changes with nuclear inclusion bodies. The testicle of Guinea Pig 10 was removed on the 9th day. Sections from this showed a very slight interstitial reaction, a few of the cells of which showed typical nuclear inclusions.

Series T—Testicle-Brain-Testicle.—April 15, 1926, Guinea Pig 11 received an inoculation into the left testicle of an emulsion made from the submaxillary glands of two full grown guinea pigs. The temperature was elevated on the 6th and 7th days, rising to 106° on the 8th day. On this day the animal was killed, the testicle removed, and placed in 50 per cent glycerol. On the following day the testicle was washed free of glycerol and emulsified in Locke's solution. 0.1 cc. of this emulsion was injected into the brain of each of two guinea pigs, Nos. 12 and 13. Beginning with the 3rd day following the injections, both animals showed an elevation of temperature, ranging between 104° and 106°. On the 7th day Guinea Pig 12 was killed. A small piece of the brain was retained for microscopic study and the remainder was placed in 50 per cent glycerol. On the 9th day following the injection, Guinea Pig 13 was killed, the brain removed, a small piece placed in Zenker's fluid for microscopic study, and the remainder placed in 50 per cent glycerol. The examination of the sections of the brains of Guinea Pigs 12 and 13 revealed, in each case, a localized meningeal reaction, and a moderate number of the cells showed characteristic changes with the nuclei containing acidophilic masses.

The brains of Guinea Pigs 12 and 13 were preserved in glycerol for 25 and 27 days, respectively. An emulsion was made from this glycerolated material on May 28 and 0.3 cc. of this was inoculated into the brain of Guinea Pig 14 and 0.1 cc. into each testicle of Guinea Pig 15. Although Guinea Pig 14 had some fever there were no marked symptoms and it was killed on the 11th day following the injection. Sections made from the brain of this animal showed no lesions of any kind.

Guinea Pig 15 had moderate fever up to 105°. It wa killed on the 11th day, and the testicles were removed. The left testicle was preserved for further inoculation and the right placed in Zenker's solution. Sections made from this testicle

showed a slight cellular infiltration and edema and in the tubules a small number of cells with nuclear inclusions were found.

It will be seen from these protocols that in seven instances it has been possible to produce lesions in two animals in series and in one other instance in three animals in series. Many variations in the technique have been made in the hope of transmitting the virus indefinitely. Transfers were made at various periods following the infection, even as early as the 2nd day. In other experiments, instead of employing the entire brain tissue for the emulsion, only scrapings from the surface of the brain were used, since the lesions containing the cells with nuclear inclusions are found only in the meningeal exudate. In other experiments it was thought possible that some stimulating or accessory substance present in the submaxillary gland might be necessary for infection and that when transfers were made from brain to brain this factor would of course be lacking. Consequently, emulsions of submaxillary glands of very young pigs were added to the brain emulsions of infected guinea pigs which were used for transfer. The results with none of these methods, however, proved successful.

Although it was not possible to transmit the virus indefinitely, the results obtained offer considerable evidence that in this condition we are dealing with an agent which reproduces itself, and, therefore, presumably is a living virus. There has been no indication so far obtained that the virus on passage tends to become more virulent. Indeed, the opposite effect has been observed. In all cases when any effect has resulted from the second transfer the lesions have been less well marked than those following the first transfer. Although the virus may be preserved, at least for short periods, in glycerol, no evidence was obtained that any increase in virulence or infectivity occurs during this time.

Infectiousness for Other Species of Animals.—Attempts have been made to reproduce the lesions in other species by the inoculation of an emulsion of the submaxillary glands of full grown guinea pigs into the brains of nine young rabbits, five young rats, and two young kittens. These animals all remained well and sections of the brains of these animals, which were killed at varying intervals, showed no lesions.

Properties of the Virus.

Thermolability of the Infectious Agent.—On February 3, a full grown guinea pig was killed and the submaxillary glands removed with sterile precautions. Histologic sections prepared from small pieces of these glands were subsequently shown to contain the specific lesions. Aerobic and anaerobic cultures of the glands made at this time remained sterile. The glands were ground thoroughly in a mortar and suspended in 5 cc. of Locke's solution. After centrifuging for a few minutes at low speed the suspension was divided into two parts; one half was heated at 54°C. for 1 hour and the other was stored on ice during this period. 0.1 cc. of the unheated suspension was then injected intracerebrally into Guinea Pigs 16, 17, and 18, and 0.1 cc. of the heated suspension was injected intracerebrally into each of the guinea pigs, Nos. 19, 20, and 21. All the pigs were less than 1 month old. One of the animals inoculated with the heated material, Guinea Pig 20, was found dead on the 2nd day after inoculation. The brain was removed and prepared for histologic examination. One of the animals which had been inoculated with the unheated material, Guinea Pig 18, was killed on the same day and sections were prepared from the brain for comparison with the sections from Guinea Pig 20.

On February 6, a marked contrast was noted between Guinea Pigs 16 and 17, inoculated with the unheated suspension, and Guinea Pigs 19 and 21, inoculated with the heated suspension. The former appeared unsteady on their feet and their hair was ruffled, whereas Guinea Pigs 19 and 21 appeared perfectly normal. All four guinea pigs were killed on the 3rd day following injection, and histologic sections were prepared from the brains. Microscopic examination of the brains of Guinea Pigs 16, 17, and 18 all showed a marked mononuclear exudate into the meninges in which numerous cells with characteristic nuclear inclusion bodies were found. On the other hand, sections from the brains of Guinea Pigs 19, 20, and 21 showed no meningeal exudate and no cells containing nuclear inclusions.

Resistance of the Virus to 50 Per Cent Glycerol.—On May 17 small pieces of the submaxillary gland of three full grown guinea pigs were placed in a small sterile bottle containing equal parts of glycerol and Locke's solution. The bottle was then stored on ice. A small piece of each gland was prepared for histologic examination and was subsequently shown to contain the specific lesion. The remainder of the glands was emulsified in the usual manner and 0.1 cc. was injected intracerebrally into each of two young guinea pigs, Nos. 22 and 23. Guinea Pig 22 was moribund on the 5th day following injection and was killed. Guinea Pig 23 was found dead on the 7th day. Microscopic study of the brains of Guinea Pigs 22 and 23 showed the usual brain lesion with typical nuclear inclusion bodies. On May 28, 11 days after placing the submaxillary glands in 50 per cent glycerol, the pieces of tissue were washed free of glycerol, ground in a mortar, and suspended in Locke's solution. After centrifuging a few minutes at low speed, 0.1 cc. was injected intracerebrally into each of the guinea pigs, Nos. 24 and 25, both less than 1 month old. Guinea Pig 25 was found dead on the 7th day following the

injection and Guinea Pig 24 was found dead on the 10th day. Microscopic study of the brains of both of these animals showed a meningeal exudate containing cells which showed typical nuclear inclusion bodies.

In another experiment in which the submaxillary gland was exposed to 50 per cent glycerol for 7 days, a similar result to that described above was obtained. In another instance the submaxillary gland was exposed to 50 per cent glycerol for 28 days. The injection of this material into the brains of young guinea pigs failed to produce the characteristic cerebral lesions.

Filterability of the Virus.—On April 15 two full grown guinea pigs were killed and the submaxillary glands removed with sterile precautions. Sections prepared from small pieces of these glands were subsequently shown to contain the specific lesion in the ducts of the glands. Aerobic and anaerobic cultures of the glands made at this time remained sterile. The glands were ground thoroughly in a mortar and suspended in a total volume of 15 cc. of Locke's solution. The suspension was centrifuged at moderate speed for 15 minutes. Half of the supernatant fluid was then filtered through a new Berkefeld N filter. The material filtered rapidly. The filter was subsequently tested and found to be impermeable to *B. coli.* 0.1 cc. of the unfiltered suspension was inoculated intracerebrally into each of three guinea pigs, Nos. 26, 27, and 28. 0.15 cc. of the filtered material was inoculated intracerebrally into each of three guinea pigs, Nos. 29, 30, and 31. All the guinea pigs were less than 1 month old. The results following injection of the unfiltered material were as follows: On April 19, the 4th day following injection, Guinea Pig 26 was found dead, No. 27 was moribund, and No. 28 seemed sick and was killed and the brain removed for histologic examination. Microscopic study of the brain of No. 28 showed an intense meningitis containing numerous cells showing typical nuclear inclusion bodies.

The results following the injections of the filtered material were as follows: Guinea Pigs 29, 30, and 31 all showed on the 2nd day, a rise in temperature ranging from 105–105.6°. On the 4th day Guinea Pig 29 had a temperature of 105.2°. On the 5th day the temperature began to drop and on the 6th day it was subnormal and the animal was killed. The brain was removed and prepared for histologic examination. Guinea Pig 31 showed a rise in temperature, ranging from 104–105° from the 4th to the 8th days. The animal was killed on the 8th day and the brain was removed for histologic examination. The temperature of Guinea Pig 30 ran an irregular course. This animal was killed on the 12th day and the brain removed. A microscopic study of the brains of these three guinea pigs showed a moderate meningitis and in every instance cells containing typical nuclear inclusion bodies were found.

In a second filtration experiment a new Berkefeld N filter was used which was tested during the course of the filtration by the addition of 0.5 cc. of an 18 hour broth culture of *B. coli* to the suspension of submaxillary gland. Cultures of the filtrate remained sterile. The same result as the one described above, was obtained.

Relation of the Infection of the Submaxillary Glands to Other Diseases of Guinea Pigs.

The only other disease affecting guinea pigs known to the writers which may possibly be related to the infectious process in the submaxillary glands is a condition described in 1911 by Römer (12). He observed sporadic cases of paralysis of the extremities in guinea pigs. By intracerebral inoculation of healthy guinea pigs with emulsions of the brains of the diseased animals he was able to transmit the infection without difficulty. After an incubation period of from 9 to 23 days the inoculated animals developed paralysis and after 2 to 10 days of severe illness, died. The brains of both the spontaneously and experimentally infected animals showed a marked infiltration of the meninges with an exudate containing many mononuclear cells and also many polymorphonuclear cells. No mention was made of the presence of cells containing nuclear inclusions.

The brain lesions in this condition resemble to some extent the lesions observed after intracerebral injection of the virus from the submaxillary glands. However, the ease with which the infection could be indefinitely transmitted and the long incubation period observed render it unlikely that the agents concerned in the two conditions are identical, though this possibility should be borne in mind.

SUMMARY AND CONCLUSIONS.

In the lesions of herpes simplex and similar conditions due to filterable viruses, cells are present which show characteristic alterations, particularly in the nucleus. The nucleus of these cells contains a mass which stains with acid dyes. Surrounding this mass is a clear space or halo, within which there are large granules staining with basic stains. These cells are little if at all enlarged.

In a few human cases, especially in infants, enlarged cells have been found which contain nuclei showing changes similar to those seen in the abnormal cells of herpes simplex.

In the ducts of the submaxillary glands of guinea pigs, Jackson observed structures which she considered to be protozoan parasites. Our own studies indicate, however, that these structures are greatly swollen epithelial cells with nuclei having the same characters as the

nuclei of the atypical cells in the lesions of herpes simplex. These cells are usually surrounded by a mononuclear cellular reaction. They were found in 84 per cent of the full grown guinea pigs examined but they were present in only three of forty-three young guinea pigs less than 1 month old. The resemblance of these cells, except as regards size, to the atypical cells present in lesions due to filterable viruses suggested that they also may be the result of an infection with a similar agent. That they are usually not present in guinea pigs less than 1 month old indicates that natural infection usually occurs after this period.

Experiments were therefore undertaken to determine whether or not an infective agent is concerned in this condition and if so to learn something of its nature. When an emulsion of the submaxillary glands of full grown guinea pigs is injected into the brains of young guinea pigs the animals have fever and exhibit symptoms of cerebral irritation. They usually die in 5 to 7 days and in sections of the brain a diffuse subacute meningitis is found. In the exudate there are large numbers of cells having all the characteristics of the abnormal cells of herpes simplex. Similar cells are present in the lesions resulting from the injection of the same emulsion into the testicle, lung, tongue, and submaxillary glands of young guinea pigs. In none of these lesions, however, are the cells greatly enlarged as they are in the lesions in old guinea pigs.

These results support the view that the lesion in the submaxillary gland of old guinea pigs is due to an infective agent. Attempts were therefore made to transmit this agent through a series of young guinea pigs. When the injections were all made into the same organ all the experiments but one gave negative results, but when the site of injection was changed at each transfer it was possible in a number of instances to reproduce the lesions through two animals in series and in one experiment through three animals in series. By modifying the technique, efforts were made to transmit the infection indefinitely but these attempts were unsuccessful. No explanation can be offered for this failure.

Studies made to determine some of the properties of the infective agent have shown that it is destroyed by heating at 54° for 1 hour, and that it is not injured by preservation in 50 per cent glycerol for as long as 11 days. After the material had remained in 50 per cent glycerol

for 28 days, however, it was found to be no longer infective. The infective agent was not held back by a Berkefeld N filter which was impermeable to bacteria. It seems probable therefore that the infective agent belongs in the group of filterable viruses, though further work will be necessary to learn more of its exact nature. These observations present additional evidence that the presence of cells with nuclear inclusions in any lesion indicates that the injury is probably due to an infective agent belonging in the group of filterable viruses.

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EXPLANATION OF PLATE 33.

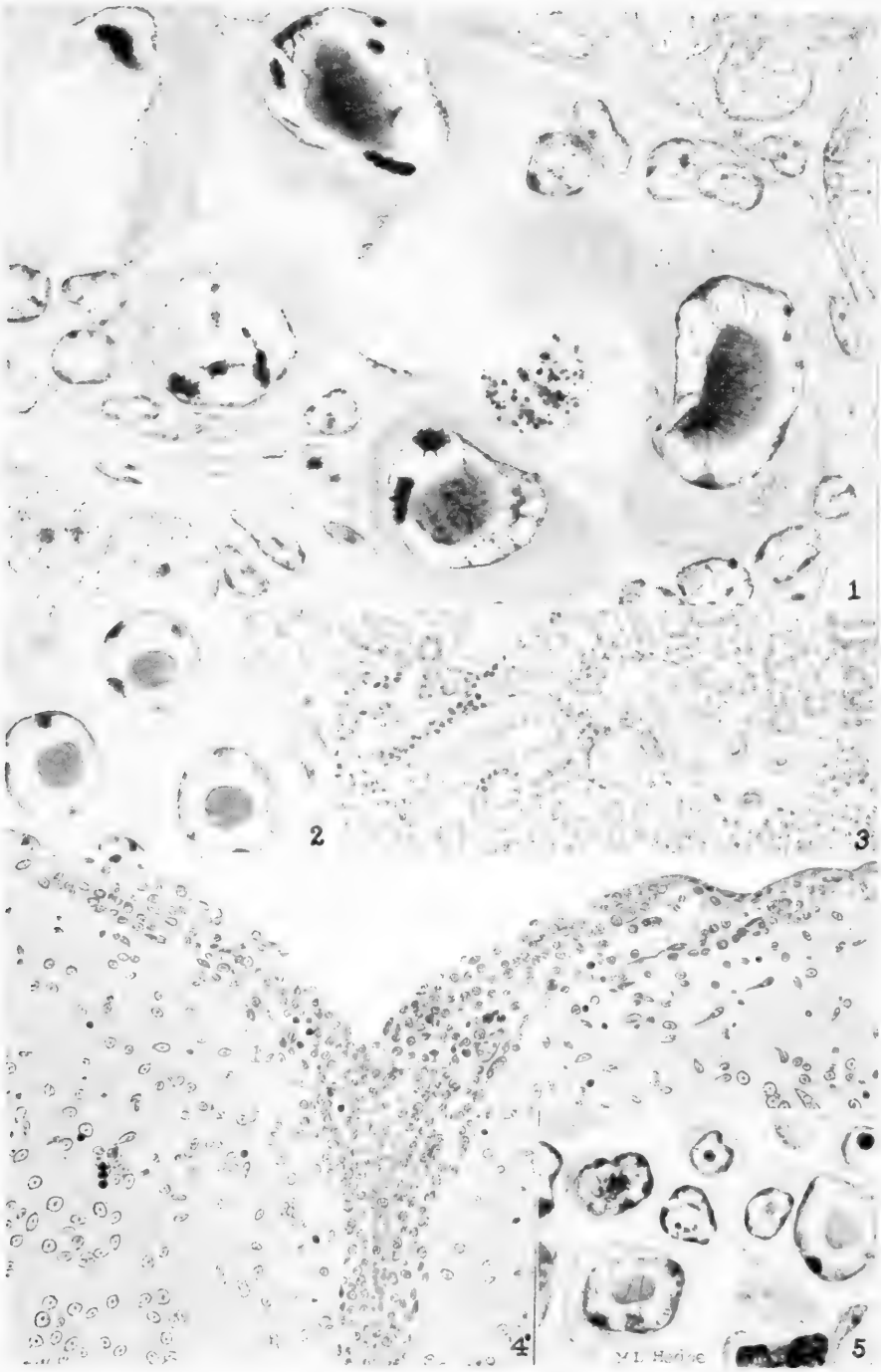
FIG. 1. Swollen epithelial cells containing nuclear acidophilic inclusions within a duct of the submaxillary gland of a full grown guinea pig. Magnification × 1700.

FIG. 2. Duct cells of smaller size containing acidophilic nuclear inclusions. This illustration was made from a section of the submaxillary gland of a young pig, exact age not known. Duct cells of this size containing inclusions have been found only rarely. Possibly these cells are in process of transformation. Magnification × 1700.

FIG. 3. Low power drawing from a section made from the submaxillary gland of a full grown guinea pig. Shows a duct swollen with epithelial cells containing nuclear inclusion bodies. There is a moderate degree of cellular reaction in the vicinity of the infected duct.

FIG. 4. Low power drawing of a section of the brain of a young guinea pig inoculated with an emulsion of the submaxillary gland of a full grown guinea pig. A well marked meningeal exudate is shown.

FIG. 5. High power (× 1700) drawing of the cellular meningeal exudate seen in low magnification in Fig. 4.



(Cole and Kuttner: Filterable virus in guinea pigs.)

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